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(57) Abstract

A method for conducting gene therapy is provided. The therapy involves using a recombinant gene that encodes an antibody that binds an antigen associated with a disease. The invention is in particular useful in providing cells with "immunity" against intracellular pathogens. Novel vectors and cell lines also are provided.

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INTRACELLULAR IMMUNIZATION

This application is a continuation-in-part of PCT patent application Serial No. PCT/US94/08448, filed on July 28, 1994, which is a continuation-in-part of U.S. patent application Serial No. 08/099,870, filed on July 30, 1993.

Field of the Invention

This invention relates to an immunological approach using gene therapy to treat infectious disease.

Background of the Invention

Advances in medicine and public health have eradicated or significantly reduced the incidence of serious illness or death caused by many pathogens. Nevertheless, infectious diseases still are responsible for many serious health problems. Some of the more problematic agents of these diseases are: viruses; mutated-resistant bacterial strains; agents that reside beyond the reach of conventional

therapeutics due to, for example, a barrier such as the blood brain barrier; and readily mutating strains.

One serious health risk has resulted from a relatively new pathogen, the human immunodeficiency virus (HIV). This virus has had devastating effects, particularly in that it opens the door to infection by a variety of opportunistic pathogens (e.g. hepatitis and tuberculosis) that usually do not pose serious health risks in HIV negative individuals. Despite billions of dollars in research, an effective treatment for HIV infection has not been discovered to date.

One common approach to treating infectious disease is the use of vaccines, which stimulate the host's immune system to be in a ready state for recognizing and destroying the pathogen. Vaccines contain immunogens that are incapable of producing the disease state, but capable of producing immunity against the pathogen. Vaccines have been very successful in protecting against infection by some pathogens, but ineffective in protecting against infection by others.

Another approach is passive immunization, which involves supplying systemically to a host antibodies that can bind the pathogen. The utility of this approach was greatly increased with the development of humanized antibodies and single-chain antibodies, both of which do not provoke an immune response by the host.

The foregoing treatments are limited in that the most active site for many diseases is within the cell, beyond the reach of antibodies. In addition, synthetic antibodies have a relatively short life, during which they are subject to serious proteolytic and other degradation.

A current experimental approach for treating infectious disease is to intracellularly express in a host a mutant form of viral protein that can strongly interfere with the replication of the wild-type virus. In cultured cells, this strategy has been successfully implemented to produce cell lines with acquired resistance to herpes

simplex virus (HSV) and HIV. Different approaches to this intracellular binding have been developed for human viral infections, including: (1) transdominant-negative mutant inhibitors; (2) specific target gene ribozymes; (3) anti-sense oligonucleotides; (4) viral receptors and receptor analogs; (5) suicide constructs; (6) virus specific inhibitory molecules; and (7) molecular decoys. To date, most of the reports of these experiments did not show completely satisfactory results.

The present invention overcomes the limitations of this prior art and vastly expands the therapeutic potential of antibodies.

Summary of the Invention

The present invention involves treating diseases by intracellular immunization. Antibody genes are delivered to cells in vectors. They "immunize" the host cells by enabling the intracellular expression of antibodies or modified antibody binding domains which are specific for important disease related antigens. These antibodies bind the antigens, thereby halting, inhibiting or retarding the development or progression of the disease. The invention can provide immunity before or after the development of the disease as well as treatment to control its severity.

According to one aspect of the invention, an improved method for conducting gene therapy is provided. The improvement involves using a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with an intracellular pathogen. Because intracellular expression of the antibody is desired, the recombinant genes of the invention preferably are prepared so as to be free of a signal sequence. In addition, the recombinant genes can be provided with localization sequences, such as a nuclear localization sequence, so that the antibodies can be targeted to desired compartments. The preferred recombinant genes encode single chain antibodies that are selectively

specific for intracellular viral antigens and that are part of an infectious agent that is replication-deficient. In accordance with other aspects of the invention, the recombinant genes encode single or multiple binding domains from one or more antibodies.

In one particularly preferred embodiment, the antibody gene is under the control of a pathogen promoter such as the HTLV-1 LTR promoter which is expression dependent upon the presence of HIV-1 tat protein, so that intracellular expression of the antibody will not occur until the cell is also infected by a pathogen that can initiate the regulatory effects of that promoter.

According to another aspect of the invention, a method for treating a subject having a disease caused by an intracellular pathogen is provided. A recombinant gene in an infectious vector is administered to the subject, the gene encoding an antibody that is selectively specific for an intracellular antigen associated with the pathogen.

According to still another aspect of the invention, an *ex vivo* treatment is provided. Cells may be isolated from a subject or derived from another source. A recombinant gene is introduced into the cells, the gene encoding an antibody that is selectively specific for an antigen associated with an intracellular pathogen, to form immunized cells. The immunized cells then are introduced into the subject.

Still another aspect of the invention involves a method for inhibiting replication of an intracellular pathogen in a cell by causing to be introduced into the cell a recombinant gene encoding an antibody that is selectively specific for an antigen associated with an intracellular pathogen.

In all of the foregoing methods, the recombinant gene can be as described above.

The invention also includes vectors containing the recombinant genes of the invention and cell lines transduced or transfected with such genes.

These and other aspects of the invention will be described in greater detail below.

Brief Description of the Drawings

Figure 1 is a diagram of the vector pT7H3-10.

Figure 2 is a diagram of the vector p4ZABVKRIDO.

Figure 3 is a diagram of the vector pSCCribo.

Figure 4 is a photograph of a gel depicting ribozyme cleavage of endogenous ABVK RNA.

Figure 5 is a diagram of the vector pLXSNCAT.

Figure 6 is a diagram of the vector pLX-GAL.

Figure 7 is a diagram of the vector pET19vHLc8.

Figure 8 is a diagram of the vector p9CESAR.

Figure 9 is a graph showing the effect of sFv-anti-rev production on syncytia formation.

Figure 10 is a graph depicting the effect of sFv-anti-rev expression on the levels of soluble p24 production.

Figure 11 is a graph showing the effect of anti-rev sFv expression on p24 antigen production in different clinically isolated HIV-1 strains.

Figure 12 is a bar graph depicting peptide mapping of human anti-tat Fab binding domains.

Figure 13 is a bar graph depicting the effect of peptide reduction on binding of human anti-tat Fab to cysteine rich domain peptides 4, 5 and 6.

Figure 14 is a bar graph depicting human anti-rev Fab binding domains.

Figure 15 is a diagram of the murine leukemia virus (MLV)-based retroviral vectors used to express the anti-rev sFv and CAT gene products.

Figure 16 is a photograph of a CAT assay depicting the patterns of gene expression of retroviral vectors cloned in human T lymphocyte cell lines. Using limiting dilution, forty cellular clones were generated from transduced CEM and Sup-T1 cells which were G418 selected for two weeks prior to being maintained in G418-free media for over two months. A total of 1 X 10^6 cells were collected from the different

clonal cell lines in which to assess the amount of CAT expression. The figure contains data generated using five representative pLXSN-transduced CEM (lanes 1-5) and Sup-T1 (lanes A-E) cellular clones.

Figure 17, comprising parts A and B, depicts the patterns of gene expression of retroviral vectors, pLXSN and pSLXCMV in human T lymphocytes.

(A) CAT expression in mixed populations of Sup-T1 and CEM cells transduced with retroviral vectors. Following transduction with 1 X 10^6 colony forming units (cfu)/ml and two weeks of G418 selection, mixed Sup-T1 and CEM cell populations were maintained in G418-free medium for two months. At various times, 1 X 10^6 cells were collected and CAT activity expressed therein was assessed. Lane 1: non-transduced Sup-T1 cells; Lane 2: non-transduced CEM cells; Lane 3: Sup-T1 cells transduced with pLXSN-CAT; Lane 4: Sup-T1 cells transduced with pSLXCMV-CAT; Lane 5: CEM cells transduced with pLXSN-CAT; Lane 6: CEM cells transduced with pSLXCMV-CAT.

(B) CAT expression in peripheral blood mononuclear cells (PBMC) transduced with retroviral vectors. In each case, 2 X 10^6 stimulated PBMC were transduced with CAT-expressing retroviral vectors and CAT activity was measured at 48 hours post-transduction. Lane A: human PBMC transduced with pSLXCMV-CAT; Lane B: human PBMC transduced with pLXSN-CAT.

Figure 18, comprising parts A, B and C, depicts an analysis of sFv gene transduction and sFv protein localization in T lymphocytes.

(A) DNA-PCR analysis of sFv gene transduction in human T lymphocytes. CEM cellular clones transduced with pSLXCMV-D8-SFv were maintained in G418-free medium for two months. A sample comprising 1 X 10^5 cells was processed for DNA-PCR analysis for the anti-rev sFv gene using the primers EAR-5 and EAR-6 (5'-CCAGATCTGATGTGCAGCTGGTGGAGTC-3'and 5'-TTGGATCCTCAGGATAGACGGGTGGGGGTG-3', respectively). PCR reaction mixtures were analyzed on 1.5% agarose gels. Lanes 1-16: Sixteen representative transduced CEM cellular

clones; Lane (-): non-transduced CEM cells; Lane (+): pSLXCMV-D8-SFv plasmid control; Lane M: PCR DNA marker. The arrow points to specific amplified bands (356 base pairs).

(B) Indirect immunostaining of the sFv protein in pLXSN-D8-SFv-transduced CEM cells. Cellular clones of anti-rev sFv-transduced CEM cells were maintained for two months in culture and were then immunostained to localize the sFv protein using rabbit anti-mouse IgG (Fab-specific). Photomicrograph depicts specific staining for sFv expression using immunofluorescence microscopy.

(C) This is a phase contrast photomicrographic image of the same field as in (B) (magnification 400 X).

Figure 19 is a series of graphs depicting HIV replication in mixed CEM and Sup-T1 cell populations transduced with anti-rev sFV. Cells were transduced with CAT- or sFv-expressing retroviral vectors. Untransduced cells served as controls. Cells were then infected with HIV_{NL4-3} or HIV_{HXB2} at multiplicities of infection (moi) of 0.024and 0.24, respectively. HIV replication was assessed by measuring p24 antigen levels in culture supernatants in an ELISA assay (Dupont). The data presented are representative of at least two separate sets of experiments.

Figure 20 comprises two graphs depicting HIV-1 infection in CEM and Sup-T1 cell clones transduced with anti-rev sFv. Either untransduced or representative CEM and Sup-T1 cell clones transduced with CAT or sFv-expressing retroviral vectors were infected with HIV_{NL4-3} at mois of 0.06 and 0.024. HIV-1 replication was assessed by measuring p24 antigen levels in culture supernatants in an ELISA assay. The data presented are representative of at least two separate sets of experiments.

Figure 21, comprising parts A and B, are photomicrographs depicting syncytium formation in sFv-transduced and control cells. Mixed cell populations of anti-rev sFv-transduced (A) and CAT-transduced (B) CEM cells were infected with HIV_{NL4-3} at an moi of 0.12. Syncytium

formation was measured and photographed on day 12 post-infection.

Figure 22 comprises two graphs depicting HIV-1 replication in anti-rev sFv transduced human PBMC. Stimulated human PBMC were transduced with the various retroviral expression vectors as shown in the figure. Mixed populations of PBMC were infected with HIV_{NL4-3} at mois of 0.24 and 0.06. HIV-1 replication was assessed by measuring p24 antigen levels in culture supernatants in an ELISA assay. These data are representative of at least two independent series of experiments using PBMC obtained from different seronegative donors.

Figure 23, comprising parts A and B, depicts cell surface CD4 antigen levels and early stages of HIV-1 expression in retroviral vector transduced and in non-transduced T-lymphocytes.

(A) CD4 antigen expression. CEM and Sup-T1 cells were transduced with retroviral vectors, selected in G418 medium for two weeks and were then incubated in G418-free medium for two months. Cells were stained for CD4 antigen using an FITC-conjugated antibody and were analyzed for CD4 expression via FACS. Non-transduced cells were used as controls in each experiment. The "% positive cells" on the Y-axis represents the percent of cells which were positive for CD4 antigen expression in each cell population.

(B) Infection of anti-rev sFv-transduced T lymphocytes with an HIV-1 construct which expresses CAT. Nine representative anti-rev sFv transduced CEM cellular clones which exhibited resistant to HIV-1 expression (two of which are illustrated in Figure 19) were infected with an HIV-1-CAT construct which encodes CAT in the *nef* open reading frame (100 ng p24 antigen equivalents of virus was used per 1 X 10^6 cells). At 24 hours post-infection, CAT activity was measured. Lanes 1-9: anti-rev sFv-transduced CEM clones infected with HIV-CAT; Lane (+): positive control for CAT activity.

Figure 24, comprising parts A and B, is a series of graphs depicting inhibition of replication of clinical isolates of HIV-1 in anti-rev transduced human PBMC.

Figure 25 is a diagram of the HIV-1 integrase gene and its protein product depicting the relative positions on the protein to which the monoclonal antibodies bind, which monoclonal antibodies form the basis of the anti-in sFv antibodies of the invention.

Figure 26 is a diagram of each of the expression vectors into which the anti-IN sFv gene is inserted.

Figure 27, comprising parts A-O, depicts the nucleotide and amino acid sequence of the monoclonal antibody genes directed against HIV-1 invertase. (A) MAb #21, anti-HIV-1 integrase heavy chain variable domain DNA sequence; (B) MAb #21 heavy chain corresponding amino acid sequence; (C) MAb #21 anti-HIV-1 integrase light chain variable domain DNA sequence; (D) MAb #7 anti-HIV-1 integrase heavy chain variable domain DNA sequence; (E) MAb #7 heavy chain corresponding amino acid sequence; (F) MAb #17 anti-HIV-1 integrase heavy chain variable domain DNA sequence; (G) MAb #17 heavy chain corresponding amino acid sequence; (H) MAb #12 anti-HIV-1 integrase light chain variable domain DNA sequence; (I) MAb #12 light chain corresponding amino acid sequence; (J) MAb #12 anti-HIV-1 integrase heavy chain variable domain DNA sequence; (K) MAb #12 heavy chain corresponding amino acid sequence; (L) MAb #33 anti-HIV-1 integrase light chain variable domain DNA sequence; (M) MAb #33 light chain corresponding amino acid sequence; (N) MAb #33 anti-HIV-1 integrase heavy chain variable domain DNA sequence; (O) MAb #33 heavy chain corresponding amino acid sequence.

Detailed Description of the Invention

The invention provides antibody-based intracellular immunity against intracellular pathogens. Recombinant antibody genes are introduced into cells. The recombinant antibody genes encode antibodies that are

selectively specific for antigens associated with the pathogen. The antibodies are expressed intracellularly and the pathogen-associated antigens are present intracellularly. The antibodies bind to the antigens and interfere with the replication of the pathogen, thereby providing the "immunity" or treatment.

The invention may be used prophylactically or therapeutically. When used prophylactically, the invention is applied to a subject that is at risk of being infected by an intracellular pathogen. When used therapeutically, the invention is applied to a subject that is known to have or that is suspected of having an infection by an intracellular pathogen.

As used herein, subject means animal. Preferred subjects are mammals, fowl and fish. Most preferred are humans, primates, dogs, cats, horses, cows, sheep, goats, pigs, rodents, chickens and turkeys.

An "intracellular pathogen" means a disease-causing organism which resides, during only part of its life cycle, within a host cell. Such pathogens include certain viruses, bacteria, fungi and protozoans. Examples include: Human Immunodeficiency Virus including, without limitation, HIV-1 and HIV-2; human T cell leukemia virus (including, without limitation, HTLV-I and HTLV-II); herpesvirus including, without limitation, herpes simplex virus type 1 (HSV-1) and type 2, varicella zoster virus; cytomegalovirus (CMV); Epstein-Barr virus (EBV); papillomavirus; hepatitis (including, without limitation hepatitis A, B, C, D and E viruses); Creutzfeldt-Jacob virus; feline leukemia virus; influenza virus; variola; rubeola; mumps virus; mycobacteria including, without limitation, *M. tuberculosis* and *M. leprae*; *Candida* including, without limitation, *Candida albicans* and *Candida tropicalis*, mycoplasma, *Toxoplasma gondii*; *Trypanosoma cruzi*; organisms of the genus *Leishmani*; and organisms of the genus *Plasmodium*.

Recombinant genes encoding antibodies with a particular binding specificity are used in the methods and

products of the invention. A recombinant gene, as used herein, is an isolated protein-coding sequence operably linked to a promoter, whereby the protein is capable of being produced when the recombinant gene is introduced into a cell. The coding region can encode a full length gene product or a subfragment thereof, or a novel mutated or fusion sequence as described in greater detail below. The protein coding sequence may be a sequence endogenous to the target cell, although according to the preferred embodiments it typically will not be a sequence endogenous to the target cell. If it is an endogenous sequence, then it is not normally expressed intracellularly within the cell or, if expressed, not at biologically significant levels. The promoter, with which the coding sequence is operably associated, may or may not be one that normally is associated with the coding sequence.

The promoters useful in constructing the recombinant genes of the invention may be constitutive or inducible. A constitutive promoter is expressed under all conditions of cell growth. Exemplary constitutive promoters include the promoters for the following genes: hypoxanthine phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase, the β-action promoter and others. In addition, many viral promoters function constitutively in eukaryotic cells. These include: the early and late promoters of SV40; the long terminal repeats (LTRs) of Moloney leukemia virus and other retroviruses, and the thymidine kinase promoter of herpes simplex virus. Other promoters are known to those of ordinary skill in the art.

Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein promoter is induced to promote (increase) transcription in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

The recombinant genes of the invention are prepared synthetically or, preferably, from isolated nucleic acids. A nucleic acid is "isolated" when purified away from

other cellular constituents, i.e., other cellular nucleic acids or proteins, by standard techniques known to those by ordinary skill in the art.

The recombinant genes of the invention can be derived from sequencing information or cell lines publicly available or may be derived from antibody producing cell lines or isolated antibody producing lymphocytes prepared according to a variety of methods. One such method involves the formation of monoclonal antibody producing hybridomas. Generally, an animal is immunized with an antigen. A fused cell hybrid then is formed between the antibody-producing cells from the immunized animal and an immortalizing cell line such as a myeloma. Alternatively, cell lines can be produced by directly immortalizing antibody-producing human lymphocytes with Epstein-Barr virus (EBV).

The recombinant genes of the invention encode antibodies that are selectively specific for intracellular antigens associated with intracellular pathogens. An antibody that is "selectively specific" for an intracellular antigen binds to that antigen, but does not bind to any appreciable degree to native intracellular constituents of the host cell. Antibodies as used herein means any portion of an antibody that retains the variable region binding specificity, including whole antibody, Fab portions, chimeric antibodies or fragments thereof including humanized and human antibodies and single chain antibodies. Single or multiple binding domains from one or more antibodies may be combined to form a chimeric antibody having the specificity of the binding domains of each antibody.

The antibodies should be selected such that they interfere with replication of the pathogen upon binding to the antigen. Antibodies that selectively bind to antigens or elements that are conserved which are critical to regulation or which are critical to replication are preferred. For example, for HIV-1, the antibodies can be selected to have specificity for important enzymes or regulatory proteins such as HIV-1 integrase, Tat, Rev and

RT. For HSV, antibodies with specificity for HSV-1 IE gene transactivator VP16 and ICP4 can be used. For hepatitis B virus (HBV), antibodies with specificity for HBV polymerase can be used. It should be understood that the foregoing are merely examples of antigens against which antibodies may be directed, and other appropriate antigens well known to or easily identified by those of ordinary skill in the art can be selected depending upon the particular pathogen of interest. Antigens can be derived from virtually any pathogen associated source, including parts, extracts or isolates of pathogens. Recombinant antigens also are useful according to the invention. Many such antigens are available in various forms from commercial sources or from depositories such as the American Type Culture Collection, Rockville, MD.

One method for selecting the antigens is DNase shotgun cleavage. This method is based upon the observation that bovine pancreatic DNA I causes double strand scission of DNA in the presence of Mn^{++}. Since cleavage is random and can be controlled by varying the enzyme concentration, temperature and/or incubation time, this method is very useful in the initial step in the generation of representative libraries having virtually any insert size range. Small random-size specific DNAs can be inserted into a vector, such as the pTOPE-T vector (Novagen, Madison, WI; U.S. Patent No. 4,952,496, the entire disclosure of which is incorporated herein by reference), for expression of fusion proteins. These bacterial expression libraries will represent substantially the epitope domain of the specific antigen.

This bacterial expression system is suitable for human antibody epitope screening. The fusion partner can ensure a high level of expression and help protect the target sequence from proteolytic degradation. Desired clones may be identified by direct screening on colony lift filters. Reagents and specific protocols are available in

kits, including the Colony Finder™ Immunoscreening Kit sold by Novagen.

Because intracellular expression is desired, the recombinant genes of the invention preferably are prepared so as to be free of a signal sequence. "Free of a signal sequence" means a deletion, mutation or modification of the signal sequence which ordinarily directs antibodies to the secretory compartments. For example, the hydrophobic amino acid core of the signal sequence for secretion can be substituted with hydrophilic residues by site directed mutagenesis. See Biocca, S. et al., "Expression and Targeting of Intracellular Antibodies in Mammalian Cells," European Molecular Biology Organization (EMBO) Journal 1: 101 (1990).

The antibodies also can be targeted to desired compartments. For example, the antibodies can be targeted to the nucleus using the nuclear localization sequence PKKKRKV of the large T antigen of SV40 virus. *Id.*

The preferred recombinant genes encode single chain FV antibodies (sFv). The sFv antibody is described in U.S. Patent No. 4,946,778 to Genex Corporation, issued August 7, 1990, the entire disclosure of which is incorporated herein by reference. sFv antibodies incorporate the complete antigen-binding Fv domain of an antibody into a single polypeptide by joining the light and heavy variable domains (vL and vH) with a linker peptide. sFv antibodies having specificity for haptens, proteins, receptors and tumor antigens have been shown to have binding affinities equivalent to those of the monoclonal antibodies from which they were derived. sFv antibodies are preferred because of their small size and their reported lack of immunogenicity.

The recombinant genes of this invention are preferably free of a signal sequence and encode an appropriate targeting sequence as desired.

The recombinant genes encoding the sFv antibodies are prepared according to methods well known to those of

ordinary skill in the art. See e.g. U.S. Patent No. 4,946,778. Briefly, hybridomas or immortalized B-cells making monoclonal antibodies to the antigens of interest are produced. Heavy and light chain cDNAs then are isolated and characterized, for example, by making DNA libraries from the foregoing immortalized cells and screening these libraries with probes for heavy and light chain clones. The heavy and light chain clones then are studied to determine the sequence of the variable domains.

The variable domains of the heavy and light chain are joined by a linker. To design a suitable linker, it is preferred to first define the extent of the variable domains. Kabat et al. defined the variable domain as extending from residue 1 to residue 107 for the lambda light chain, to residue 108 for kappa light chains and to 113 for heavy chains. (Kabat, E.A., "Sequencing of Protein of Immunologic Interest", U.S. Department of Health and Human Services, U.S. Government Printing Office, 1987). The linker described in U.S. Patent No. 4,704,692 (incorporated herein by reference) can be used to join the domains. This linker was designed using a computer program that matched the ends of the variable domain with all possible structural fragments found in the protein DATABank. It should be understood that the design of a suitable linker is within the knowledge of those of ordinary skill in the art. (See e.g. U.S. Patent No. 4,946,778; Methods: A Companion to Methods in Enzymology Vol. 2, No. 2, April 1991, pp 97-105).

The sFv antibody may be constructed by joining either vL as the N-terminal domain followed by the linker and vH. A preferred linker for constructing a vH-linker-vL sFv antibody is the single linker designed by Huston et al., a (gly-gly-gly-ser) 3 linker designed to bridge the 3.5 nm gap between C terminals of vH and the N terminals of vL, without exhibiting any propensity for ordered secondary structure (Huston, J.S. et al., Proc. Natl. Acad. Sci. USA 85 pp 5879-5883, 1988). Minor modifications of this linker

design appear to have little effect upon the in vivo performance of an sFv antibody.

The sFv gene then can be engineered to encode an identification signal such as the Tat nuclear translocation signal. Because there exist specific antibodies to this signal, anti-idiotype antibody will not be necessary for immunostaining to determine sFv expression and intracellular location.

The sFv recombinant gene may be placed in a cassette that provides for efficient introduction into a cell and subsequent selection, for example, by G418 or gpt selection. After selection, cells can be evaluated for DNA, RNA and protein expression using DNA-PCR, RT-PCR and radioimmune precipitation, as well as immunostaining.

The recombinant genes of the invention are introduced into cells using vectors. Almost any delivery vector can be used, although the vector selected will depend upon the particular disease being treated, the particular form of treatment, whether the treated cells are replicating cells and other factors known to those of ordinary skill in the art.

Genetic material can be introduced into a cell by, for example, transfection or transduction. Transfection refers to the acquisition by a cell of new genetic material by incorporation of added DNA. Transfection can occur by physical or chemical methods. Many transfection techniques are known to those of ordinary skill in the art including: calcium phosphate DNA co-precipitation; DEAE-dextran DNA transfection; electroporation and cationic liposome-mediated transfection. Transduction refers to the process of transferring nucleic acid into a cell using a DNA or RNA virus. Suitable viral vectors for use as transducing agents include, but are not limited to, retroviral vectors, adeno associated viral vectors and Semliki Forest virus vectors.

The treatment of cells may be *in vivo* or *ex vivo*. For *ex vivo* treatment, cells are isolated from an animal (preferably a human), transformed (i.e. transduced or

transfected *in vitro*) with a vector containing a recombinant gene of the invention, and then administered to a recipient. Procedures for removing cells from animals are well known to those of ordinary skill in the art. In addition to cells, tissue or the whole or parts of organs may be removed, treated *ex vivo* and then returned to the patient. Thus, cells, tissue or organs may be cultured, bathed, perfused and the like under conditions for introducing the recombinant genes of the invention into the desired cells. The preferred treatment is *ex vivo* and the preferred cells for *ex vivo* treatment are stem cells.

For *in vivo* treatment, cells of an animal, preferably a mammal and most preferably a human, are transformed *in vivo* with a vector containing a recombinant gene of the invention. The *in vivo* treatment may involve systemic treatment with a vector such as intravenously, local internal treatment with a vector such as by perfusion, topical treatment with a vector and the like. When performing *in vivo* therapy, the preferred vectors are based on noncytopathic eukaryotic viruses in which nonessential or complementable genes have been replaced with the gene of interest. Such noncytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. Retroviruses have recently been approved for human gene therapy trials. Most useful are those retroviruses that are replication-deficient (i.e. capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture

media, and infection of the target cells with viral particles) are provided in Kriegler, M. "Gene Transfer and Expression, a Laboratory Manual", W.H. Freeman Co., New York (1990) and Murry, E.J. e.d. "Methods in Molecular Biology", Vol. 7, Humana Press, Inc., Cliffton, New Jersey (1991). Generation of sFv-encoding retroviruses is described herein.

A preferred virus for certain applications is the adeno-associated virus, a double-stranded DNA virus. The adeno-associated virus can be engineered to be replication deficient and is capable of infecting a wide range of cell types and species. It further has advantages such as: heat and lipid solvent stability, high transduction frequencies in cells of diverse lineages, including hemopoietic cells; and lack of superinfection inhibition thus allowing multiple series of transductions. Recent reports indicate that the adeno-associated virus can also function in an extrachromosomal fashion.

Recombinant genomes that are between 50% and 110% of wild-type adeno-associated virus size can be easily packaged. Thus, a vector such as d13-94 can accommodate an insertion of 4.7kb in length. A modified sFv will be approximately 1 to 1.5kb in length, and therefore the adeno-associated virus may be an ideal delivery system.

In one preferred embodiment, an anti-HIV-1 sFv (pAVsFv-Integ) can be constructed by removing all endogenous coding sequences (bases 190-4034) from an infectious molecular clone of an adeno-associated virus (pAV1 from ATCC, Rockville, MD.). The RSV long terminal repeat (LTR) driven sFv and the Neo gene under the control of the SV40 early promoter is then inserted into this virus.

Semliki Forest virus vectors which are useful as transducing agents include, but are not limited to, pSFV1 and pSFV3-*lacZ* (Gibco-BRL). These vectors contain a polylinker for insertion of foreign genes therein which is followed by a series of stop codons. The gene of choice is inserted into the polylinker region and viruses are generated using the *in vitro* packaging helper virus system

also provided by Gibco-BRL. Following the directions of the manufacturer and the disclosure contained herein, it is a relatively simple matter for one of skill in the art to generate Semliki Forest virus vectors capable of expressing the sFvs of the invention.

Transgenic animals also may be produced according to the invention. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats.

A variety of methods are available for the production of transgenic animals associated with this invention. DNA can be injected into the pronucleus of a fertilized egg before fusion of the male and female pronuclei, or injected into the nucleus of an embryonic cell (e.g., the nucleus of a two-cell embryo) following the initiation of cell division (Brinster et al, Proc. Natl. Acad. Sci. USA 82: 4438-4442, 1985). Embryos can be infected with viruses, especially retroviruses, modified to carry the nucleotide sequences of the invention which encode intracellularly expressed antibodies.

Pluripotent stem cells derived from the inner cell mass of the embryo and stabilized in culture can be manipulated in culture to incorporate nucleotide sequences of the invention. A transgenic animal can be produced from such cells through implantation into a blastocyst that is implanted into a foster mother and allowed to come to term.

Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan Sprague Dawley (Indianapolis, IN), etc.

The procedures for manipulation of the rodent embryo and for microinjection of DNA into the pronucleus of the zygote are well known to those of ordinary skill in the art (Hogan et al., supra). Microinjection procedures for

fish, amphibian eggs and birds are detailed in Houdebine and Chourrout, Experientia 47: 897-905 (1991). Other procedures for introduction of DNA into tissues of animals are described in U.S. Patent No. 4,945,050 (Sandford et al., July 30, 1990).

By way of example only, to prepare a transgenic mouse, female mice are induced to superovulate. Females are placed with males, and the mated females are sacrificed by CO_2 asphyxiation or cervical dislocation and embryos are recovered from excised oviducts. Surrounding cumulus cells are removed. Pronuclear embryos are then washed and stored until the time of injection. Randomly cycling adult female mice are paired with vasectomized males. Recipient females are mated at the same time as donor females. Embryos then are transferred surgically.

The procedure for generating transgenic rats is similar to that of mice (see Hammer et al., Cell 63: 1099-1112, 1990).

Methods for the culturing of embryonic stem (ES) cells and the subsequent production of transgenic animals by the introduction of DNA into ES cells using methods such as electroporation, calcium phosphate/DNA precipitation and direct injection also are well known to those of ordinary skill in the art. See, for example, Teratocarcinomas and Embryonic Stem Cells, A Practical Approach, E.J. Robertson, ed., IRL Press (1987).

In cases involving random gene integration, a clone containing the sequence(s) of the invention is co-transfected with a gene encoding resistance. Alternatively, the gene encoding neomycin resistance is physically linked to the sequence(s) of the invention. Transfection and isolation of desired clones are carried out by any one of several methods well known to those of ordinary skill in the art (E.J. Robertson, *supra*).

DNA molecules introduced into ES cells can also be integrated into the chromosome through the process of homologous recombination (Capecchi, Science 244: 1288-1292,

1989). Methods for positive selection of the recombination event (i.e., neo resistance) and dual positive-negative selection (i.e. neo resistance and gancyclovir resistance) and the subsequent identification of the desired clones by PCR have been described by Capecchi, *supra* and Joyner et al., Nature 338: 153-156 (1989), the teachings of which are incorporated by reference herein. The final phase of the procedure is to inject targeted ES cells into blastocysts and to transfer the blastocysts into pseudopregnant females. The resulting chimeric animals are bred and the offspring are analyzed by Southern blotting to identify individuals that carry the transgene.

Procedures for the production of non-rodent mammals and other animals have been described by others. See Houdebine and Chourrout, supra; Pursel et al., Science 244: 1281-1288 (1989); and Simms et al., Bio/Technology 6: 179-183 (1988).

In the experiments described herein, the ability to transduce various human T lymphocytic cells with retroviral shuttle vectors expressing an anti-rev sFv thereby rendering these cells relatively resistant to high levels of HIV-1 expression is demonstrated. In addition, protection of human PBMC against wild type HIV-1 replication is also demonstrated using this approach. Since only a few genetic therapeutic modalities have demonstrated efficacy in primary human cells (Woffendin et al., Proc. Natl. Acad. Sci. USA 91:11586-11590, 1994; Leavitt et al., Human Gene Therapy 5:1115-1120, 1994), these data suggest that this technology may be useful for in HIV-1 infected individuals.

Intracellular immunization of humans against HIV-1 may be accomplished by recovery of cells from the individual followed by *ex vivo* transduction of these cells with a construct of interest. Cells which are transduced are then returned to the individual by re-infusion. The data presented herein provide a viable approach for the immunization of primary cells using constructs encoding anti-HIV-1 sFv.

The use of recombinant antibody fragments, expressed intracellularly is especially promising as an approach to inhibit HIV-1 replication. The wide diversity and exquisite antigen-binding specificity of antibody repertoires allows for timely and efficient targeting of various critical HIV-1 specific proteins.

While intracellular sFv expression may itself induce an immune response to small quantities of the foreign protein, it is important to note that unlike complete antibodies, sFv molecules are poorly immunogenic (Winter et al., Nature 349:293-299, 1991).

The experiments described below demonstrate the ability of an intracellular antibody fragment directed to a critical retroviral regulatory protein to significantly inhibit HIV-1 replication in human T-lymphocytes, in human PBMC, and to be effective against both cloned laboratory virus strains and against clinical isolates of HIV-1. Thus, these studies establish the utility of this approach in immunizing cell-types which represent major *in vivo* cellular reservoirs for HIV-1. Recent data demonstrating that *ex vivo* transduced human lymphocytes migrate to lymph nodes is also evidence of the utility of *ex vivo* transduction protocols for inhibition of HIV-1 infection in humans. In addition, since there exists a high rate of virion and CD4-positive lymphocyte turnover in HIV-1-infected-individuals, use of "HIV-1 resistant cells", in combination with effective anti-retroviral pharmaceutical agents may arrest this rapid virion turnover (Wei et al., nature 373:117-122, 1995; Ho et al., Nature 373:123-126, 1995).

The invention should not be construed to be limited to the retroviral vectors disclosed nor be limited to the use of an anti-rev sFv. As noted above, other transduction vehicles capable of expressing anti-HIV-1 sFvs, including adeno-associated virus vectors and Semliki Forest virus vectors may also be used (Muzyczka, Curr. Top. Microbiol. Immunol. 158:97-129, 1992). Further, as is also described herein, viral vectors comprising sFvs directed

against other HIV-1 encoded proteins, for example, the HIV-1 invertase (in) are also useful for intracellular immunization against HIV-1 infection. Yet other viral proteins which may be useful as intracellular immunization agents include sFvs directed against any other HIV-1 specific function which when expressed in a cell expressing an anti-HIV antibody specific for that gene either diminishes or ablates or otherwise protects the cell and subsequently the human against infection with HIV-1.

The following examples are illustrative of the methods and compositions of the invention and are not meant to limit the invention in any way.

Examples

Example 1: RNA Isolation, cDNA Synthesis and Amplification of Vh and Vl

RNA was prepared from 5 x 10^7 hybridoma cells. The total RNA was used for first strand cDNA synthesis using 17 bp poly-T mixed with either the Vh or Vl 3' primer at 42°C for 1 hour in 50 μl reaction mixture containing 100 μg of RNA and AMV reverse transcriptase 100 Units, with a standard buffer system. For amplification of vL and vH, 5 μl of cDNA was subjected to 35 cycles of PCR using reagents, as per the manufacturer's instructions (Gene Amp. Perkin-Elmer/Cetus), in two separate tubes with 1 μM each with either vL-5' or vH-5' primer (obtained from Novagen, Inc., Madison, WI). Each PCR cycle consisted of denaturation at 94°C for 1 minute annealing at 50°C for 90 sec, and polymerization at 72°C for 2 minutes, and finally a 10 minute extension. The amplified vL and vH fragments were purified on 1.5% low-melting agarose with the Promega PCR magic purification kit (Madison, WI).

Taq polymerase-amplified PCR products were directly ligated into a modified pT7Blue(R) vector (Novagen), pT7H3-10, Figure 1, which carries extra T's at the 5' end. After transformation into the Novablue E. coli strain (Novagen), recombinants were selected on X-gal

plates. For each of the cDNA, 40 colonies were picked up for mini preparation of plasmid for further enzymatic digestion to check the size of the insert. Plasmids were further prepared for DNA sequencing. All of the plasmids were sequenced on a 373A ABI automatic DNA sequencer (ABI, Foster City, CA). Finally, the plasmids were confirmed using the USB Sequenase Kit (United States Biochemical, Cleveland, OH).

The foregoing was applied to a murine hybridoma making a monoclonal antibody against HIV-$1_{(IIIB)}$ rev.

For the vL chain, the original insert DNA sequence was confirmed to be a mutant endogenous K chain by computer homology searching. Because sp0/2 myeloma cells have endogenous K chain expression, the Complementarity Determining Region (CDR) sequence specific for endogenous K chains is used for K chain PCR recombinant plasmid screening to eliminate the contamination of this K chain from the recombinant plasmids. Only less than 5% of the plasmid do not contain this K chain and those plasmids are DNA sequenced.

For each of the cDNA fragments, at least 3 different colonies are sequenced to confirm sequence. Specific primer targeting-CDR derived from those cDNA sequences are designed to repeat RT-PCR for each of the parent hybridomas to confirm the sequence.

Specific protocols to eliminate aberrant endogenous K chains permit quickly obtaining larger numbers of different Ig K genes.

Two methods have been developed to eliminate endogenous ABVK chains. The first method is to eliminate ABKV RNA background by cleaving ABKV RNA directly with the ribozyme RNA system. This reduces the ABKV RNA RT-PCR background and enhances the specific Ig light chain RNA signal for cloning. Specifically;

A 62 bp ABKV ribozyme DNA fragment was synthesized by PCR and inserted into vector pGEM4Z at the HindIII-BamII site to form the plasmid p4ZABVKRIBO (Figure 2). After the

plasmid is linearized by BamHI digestion, the specific ABKV ribozyme can be synthesized with T7 RNA polymerase *in vitro* as follows:

Heat using 2 μg of the linearized plasmid for 3 minutes at 75°C and then cool on ice. Add the following reagents:

4 μl 5x transcription buffer
1 μl RNase inhibitor (40u/μl)
2 μl 100mM dithiothreitol (DTT)
4 μl 250 μM NTP
1 μl RNA polymerase
Add DEPC-H_2O to final volume of 20 μl.

Incubate 37°C for 1 hour. After transcription, the reaction mixture is treated with 1 μl of RQ1 DNase (5u/μl) at 37°C for 39 minutes.

This is followed by phenol/chloroform extraction and ethanol precipitation. The specific Ribozyme RNA can be resuspended in diethylpyrocarbonate (DEPC) treated water and stored at -70°C.

Total or polyA RNA, which is extracted from the hybridomas and resuspended in 5 μl DEPC water, is mixed with 4 μl ABKV ribozyme RNA. This mixture is heated to 75°C for 5 minutes, quickly cooled down on ice and resuspended in 4x RT buffer (200mM tris HC, pH 8.3, 200 mM KCl, 40mM MgCl2, 2mM spermidine, 40 mM DTT. It is then incubated at 37°C for 30-60 minutes. 5 μl of the mixture can be used for the standard PT-PCR for the Ig light chain.

A second method for eliminating the endogenous ABVK chain is as follows:

Manipulation of RNA for extended time periods or in multiple step processes may cause dramatic RNA degradation and affect the efficiency of RT-PCR. To further improve on the above ABVK ribozyme system, we have inserted this 62 bp ABVK ribozyme fragment into a new plasmid pSCCribo which may be transfected into the packaging cell line PA317 to produce an infectious but replication deficient virus (Figure 3). The supernatants containing the

virus can be used to introduce the ABVK ribozyme into any hybridoma cell line with high efficiency.

Typically, 10 ml of cell free viral supernatants (10^6 - 10^7 cpu/ml) is used to infect 5-8 x 10^6 hybridoma cells in a 20 ml total volume with 8 μg/ml polybrene at 37°C in CO_2 incubator for 24 hours. Then cells are washed twice with serum free medium and fresh medium added, with 10% FCS, and 500 μg/ml G418. This selection carried out for 1 week. RNA can then be extracted directly from hybridoma cells due to the high level of expression of the CAT-ABVK ribozyme RNA (CAT RNA is very stable in the cells). The CAT-ABVK ribozyme can specifically target the endogenous ABVK RNA resulting in cleavage. This dramatically reduces the ABVK RNA background and enhances the antigen specific hybridoma Ig light chain for RT-PCR (See Figure 4, Gel).

An alternative way to obtain vL fragments is to use commercially available filamentous phage vector systems. The vector systems can concurrently produce free Fab fragments and Fab displayed on the surface of bacteriophage via a vHC_{H1}-pIII fusion protein. When expressed in a supo (non-suppressor) strain of E. coli, free Fab can be produced. Antibody Fab fragments are secreted into culture medium at high concentration, because vH and vL are found to accumulate in the periplasmic space.

Example 2: E. Coli bacteriophage expression system for Fab

The bacteriophage expression is carried out as specified in Barbas and Lerner, Methods: A Companion to Methods in Enzymology 2: 119-124 (1991). Briefly, RT-PCR DNA encoding the Fd is inserted into a phage vector and transformed into host bacteria. RT-PCR light chain DNA fragments from the same hybridoma are then inserted into the pComb3 vector. Following bacterial transformation, the combinatorial libraries are treated to prepare phagemid.

Solid phase selection (panning) of the Fab against the antigen of interest proceeds as follows: Microtiter wells were coated with 9.5 μg of purified E. coli recombinant antigen (such as HIV-1-RT, Tat or Rev),

overnight at 4°C. Wells are blocked with bovine serum albumin (3% BSA in PBS) for one hour at 37°C, incubated with phage libraries (typically >10^{11} colony-forming phage per well), washed and eluted. The selected phage are then allowed to infect E. coli XL-IBlue cells and used to prepare a new phage stock by infection with the helper phage VCSM 13 (both from Stratagene, La Jolla, CA). For repanning against the same antigen (up to 4 more cycles) this procedure was repeated three or four times.

The culture of phagemid containing XL-IBlue cells from the last panning against antigen is split in two and one half is packaged with CSM13 helper phage, the other half used to prepare phagemid DNA. Phagemid DNA is digested with restriction enzymes to excise the geneIII coding for the phage cap protein allowing the Fabs to be expressed in soluble form. The religated DNA is retransformed into XL-IBlue cells and clone supernatants screened for Fab production by ELISA using microtiter wells coated with 0.1 μg of antigen, followed by clone supernatant, then goat anti-human $F(ab)_2$ conjugated to alkaline phosphatase, then alkaline phosphatase substrate.

Positive clones are then tested for specificity against a umber of different antigens (viral and human) by ELISA and phagemid DNA prepared from each clone.

The above methods have been applied to production of human viral neutralizing Fab to HIV-1, respiratory syncytial virus (RSV), CMV, HSV-I and II (Burton et al., PNAS 88: 10134 (1991); Barbas et al., PNAS 89: 10164, 1992; Williamson et al., PNAS 90: 1993).

Example 3: Retrovirus construction using CAT and in vitro expression of CAT

In order to demonstrate that antivirus sFv can function at different levels in different types of cells, U1 and ACH2 cells were selected to test LXSN expression function. The U1 cell line, a U937-derived HIV-1 infected clone, has been used as a model for viral latency, and the effects of monocyte-specific cytokines on the induction of HIV-1 expression were studied in this model system. ACH2 is

derived from an infected T-lymphocyte line which has one copy of provirus integration while U1 cells have two proviral copies. Both cell lines produce very low levels of HIV-1 P24 expression and act as an HIV-1 latency state. With different stimulation, such as PMA or TNF-α, both of these cells will increase HIV-1 p24 by more than 1000-fold in 48 hours, and will produce infectious functional virus. Those cell lines provide good cell line model systems which not only represent both T-lymphocyte and macrophage type cells but also represent most of the HIV-1 infected cellular populations.

A 734 bp CAT fragment was inserted into the pLXSN vector (MuLV retrovector). This pLXSNCAT plasmid (Figure 5) was transfected into the packaging cell line PA 317 and selected in G418 (1 mg/ml). 1 x 10^6 PA317 cells were plated in 100mm dishes in 10 ml Dulbecco's Modified Eagles Medium (DMEM) + 10% Fetal Calf Serum (FCS) one day before transfection. Three hours before transfection, the 10 ml of medium was replaced with 10 ml of fresh prewarmed medium. 20 μg pLXSNCAT was transfected into pA317 cells. After an additional 48 hours, the cultured medium containing the virus was collected and passed through a 0.45 μm filter to prepare cell free virus. After determining the infectious titer of the virus (cfu/ml), the medium was mixed with 3 x 10^6 U1 or ACH2 cells with 8 μg/ml polybrene to help increase efficiency and incubated for 12-16 hours. Cells were then washed twice with serum free medium and resuspended in 10 ml RPMI 1640 with 10% FCS for further culture. From this test, we can detect CAT activity after 48 hours transfection without non-specific stimulation of HIV replication (i.e. maintained at the same level of p24 as prior to superinfection).

In order to test actual infection efficiency, the 3.2 kb of E. coli β-galactosidase was inserted into the same vector, pLXSN, to construct the pLX-GAL (pLXNLacZ-13) plasmid (Figure 6). The same protocol as above was used (s

for transfection of U1 and ACH2 cells) to produce virus carrying the β-galactosidase.

After transduction into U1 and ACH2 cells, cells were stained with X-gal substrate so that cells carrying the plasmid became blue due to expression of β-galactosidase. By counting the blue cells under the microscope, U1 and ACH2 efficiency may be measured (usually more than 70% cells/per infection).

To test for long term levels of expression following transduction, with the CAT expression virus, both U1 and ACH2 cell lines were maintained in G418 selection for more than 6 months. Data shows that in ACH2 cells, CAT activity is maintained at the same level of expression for the long term. In U1 cells, CAT expression in most cases is maintained only for 2 weeks and then completely shuts off. Further tests show that after PMA stimulation, CAT activity remains at the same level but HIV-1 expression occurs as in the parent line. These data show that this DNA delivery model system can be used for delivering sFv.

Example 4: Plasmid vector construction using sFv with anti-rev activity and expression in Hela-T4s

A single chain sFv anti-rev antibody was constructed consisting of variable domains of the heavy (vH) and light (vL) chains of a murine monoclonal antibody against HIV-1_{IIIB}) rev (the "parent antibody").

Protocols for constructing the vH and vL regions are as follows:

After sequencing the anti-rev vH and vL cDNA, the CDR region was compared by computer with the published Ig protein sequences. The full length sequence was then designed. First two synthesized oligonucleotides were used to create a linker DNA fragment with ApaI-BgIII sites. This was then cloned into pT7/Blue (R) vector in order to determine the DNA sequence. vH and vL were then reamplified with two new pairs of oligonucleotides with suitable enzyme sites at both ends, cloned into pT7/Blue (R). After verifying the DNA sequence, the linker DNA, N-GGGGSGGGGSGGGGS-C (Sequence I.D. number 2), was inserted

into the ApaI and the BgIII site to connect the vH and vL DNA sequence to make a full length sFv fragment. Following digestion with NdeI-BamHI, the full length sFv DNA sequence was inserted into the E. coli expression vector, pET19b to construct plasmid pET19bHLc8 (Figure 7). Transformation into E. coli BL21 (DE3) allowed the expression of the sFv protein. A 10 histidine (HIS) amino acid domain was located on the end terminal of the sFv protein. The His-Tag sequence binds to the divalent cation (Ni^{2+}) immobilized on a His binding metal chelation resin allowing purification by Ni^{2+} affinity chromatography.

The DNA sequence of the sFv anti-rev was determined to be as follows (Sequence I.D. Number 1):

ATGGGCCATC ATCATCATCA TCATCATCAT CATCATAGCA
GCGGCCATAT CGACGACGAC GACAACCATA TGTTGGTGCT
GACGTTCTGG ATTCCTGCTT CCAGCAGTGA TGTTGTGATG
GCCCAAACTC CACTCTCCCT GCCTGTCAGT CTTGGACATC
AAGCCTCCAT CTCTTGCATA TCTAGTCAGA GCCTTGTACA
CAGTAATGGA AACACCTATT TACATTGGTA CCTGCAGAAG
CCAGGCCAGT CTCCAAAGCT CCTGATCTAC AAAGCTTCCA
ACCGATTTTC TGGGGTCCCA GACAGGTTCA GTGGCAGTGG
ATCAGGGACA GATTTCACAC TCAAGATCAG CAGAGTGGAG
GCTGAGGATC TCCCAGTTTA TTTCTGCTCT CAAAGTACAC
ATTTTCCGTG GACGTTCGGT GGAGGCACCA AGCTGGAAAT
CAAACGGGCT GATGGGCCCG GTGGGGGCGG TTCGGGTGGC
GGGGGCTCGG GCGGGGGTGG CTCAGAGCTC GGCAGATCTG
ATGTGCAGCT GGTGGACTCT GGGGGAGGGT TAGTGCAGCC
TGGAGGGTCC CGGAAACTCT CCTGTGCAGG CTCTGGATTC
ACTTTGACTA GGTTTGGAAT GCACTGGGTT CGGCAGGCTC
CAGAGAAGGG GCTGGACTGG GTCGCATACA TTAGTAGTGG
GAGTAGTACC CTCCACTATG CAGACACAGT GAAGGGCCGA
TTCACCATCT CCAGACACAA TCCCAAGAAC ACCCTGTTCC
TGCAAATGAA ACTACCCTCA CTATGCTATG CACTACTGGG
GTCAAGGAAC CTCAGTCACC GTCTCCTCAG CCAAAACGAC
ACCCCCACCC GTCTATCCTG A

Rev is one of the essential regulatory proteins of Human Immunodeficiency Virus. It is a 19kD phosphoprotein localized primarily in the nucleolus/nucleus, and acts by binding to Rev Responsive Element (RRE) and promoting the nuclear export, stabilization and utilization of the viral mRNA's containing RRE.

The binding affinity of the sFv anti-rev produced in E. coli was then determined by using an ELISA (Enzyme Linked Immunoassay) utilizing recombinant rev conjugated

with biotin. The binding affinity was approximately 10^{-7} which was comparable to the affinity of the present antibody.

The binding efficiency was determined as follows:

Purified E. coli derived sFv anti-rev was diluted in Phosphate Buffered Saline (PBS) solution at 200 μg/ml. ELISA plate wells were coated with 200 μl per well of this solution, overnight at 4°C. The same concentration of BSA/PBS was used for coating control wells. Wells were washed once with PBS and blocked by the addition of 10% BSA/PBS, 200 μl/well. After blocking for 1 hour at 37°C wells were washed three times with 0.5% Tween 20/PBS. 100μl of biotin conjugated-rev dilutions (serial 5 fold 50 μg/ml to 16 ng/ml) were added to the wells and the plates incubated for two hours at 37°C. Wells were washed 3 times with 0.5% Tween 20/PBS. 100 μl of avidin-labelled with Horseradish Peroxidase (HRP) was added to wells and incubated for 15 minutes at 37°C. Washing was repeated 3 times as above. 100 μl of color substrate solution was added to each well. After incubation at room temperature for 30 minutes the reaction was stopped by the addition of 100 μl of 4M sulfuric acid. Optical density was then read at 495 nm.

Calculation of the native rev protein was performed as follows:

rev. MW=13019.855 (117 amino acids)

E. coli derived rev has an additional 12 aa leading sequence (Sequence I.D. number 3).

Leading sequence: MRAKLLGIVLTT=1485.4

Actual molecular weight of the E. coli derived rev is = 14505.25

Therefore 14505 μg/ml = 1μM/ml= 10^{-6}M.

The data from ELISA indicates binding of rev to HLc8 protein between 2 μg and 4 μg per ml. Therefore binding affinity is approximately 7.25 x 10^{-6}M.

The effect of sFv-anti-rev production on the levels of soluble p24 expressed is demonstrated in the graph in Figure 10.

In combination, the results of syncytia formation and p24 production show that the expression of sFv anti-rev resulted in a decrease in HIV expression of approximately 80% as compared with the Hela-T4 control. This proves that sFv antibodies can be expressed intracellularly to inhibit HIV.

The sFv was then cloned into a plasmid vector ($pREP_4$, Invitrogen, San Diego, CA) which allows for expression of the sFv in mammalian cells. The sFv gene was inserted into XhoIBamHI site on the vector. It was driven by the RSV-LTR promoter. The HIV-Tat nuclear translocation signal DNA was cloned by PCR. The HIV Tat cDNA was amplified with two oligo primers and was then ligated into pT7 Blue(R) vector and sequenced. The amino acid sequence of the signal is: N-GRKKRRQRRRAHQN-C (Sequence I.D. number 4). The corresponding DNA sequence is: 5' GGC AGG AAG AAG CGG AGA CAG CGA CGA AGA GCT CAT CAG AAC AGT CAG ACT 3' (Sequence I.D. number 5).

It was inserted into the pETHLC8 SacI-BgIII site. Then the XhoI BamHI fragment was inserted into pP9 pREP9 (Neo resistant) vector to form plasmid p9CESAR (Figure 8). Hela cells expressing CD4 (Hela-T4's) were then transfected with the $pREP_4$-sFv construct which also contained tk driven neomycin resistant gene as a marker. After transfection, the Hela-T4's were incubated with neomycin (G418) to enrich the population of sFv expressing cells.

sFv expressing cells and non-transfected Hela-T4's (as a control) were then infected with a high titer of HIV-$1_{(HXB2)}$, vigorously washed and incubated for 10 days to determine the effect of sFv anti-rev production on HIV infection. This effect was measured in terms of (a) syncytia formation and (b) the levels of soluble p24 antigen.

The effect of sFv-anti-rev production on syncytia is demonstrated in the graph shown in Figure 9.

Due to the high rate of mutation of the HIV-1 genome it is important that therapies be effective on different clinically isolated strains. Figure 11 shows that the sFv specifically binds a highly conserved rev domain. The HeLa T4 cells expressed sFv resistance to all of the tested clinically isolated strains of HIV-1.

The invention can clearly be translated to other diseases caused by other pathogens and diseases associated with the elevated expression of proteins, such as cancers.

Example 5: Human Lymphocyte RNA Preparation

Five milliliters of bone marrow was removed by aspiration from an long term asymptomatic HIV-1 positive donor. Immediately, 10 ml of 3M guanidium isothiocyanate containing 71μl of 2-mercaptoethanol was added and then RNA was prepared by standard methods.

Example 6: Phagemid Library Construction

Total RNA (typically 10 μg) was reverse-transcribed as described by Burton, et al. *Proc. Natl. Acad. Sci., U.S.A.*, 88, 10134-10137 (1991), incorporated by reference herein in its entirety and γ1 (Fd region) and κ chains were amplified by PCR. The resulting γ1 heavy chain DNA was cut with an excess of the restriction enzymes Xho I and Spe I and typically about 350 ng was ligated with 2 μg of Xho I/Spe I-linearized pComb3 vector (isolated by agarose gel electrophoresis) in a total volume of 150 μl with 10 units of ligase (BRL) at 16°C overnight. Following ligation, DNA was precipitated at -20°C for 2 hr by the addition of 2 μl of 2% (wt/vol) glycogen, 15 μl of 3 M sodium acetate (pH 5.2), and 330 μl of ethanol. DNA was pelleted by microcentrifugation at 4°C for 15 minutes. The DNA pellet was washed with cold 70% ethanol and dried under vacuum. The pellet was resuspended in 10 μl of water and transformed by electroporation into 300 μl of *Escherichia coli* XL1-Blue. After transformation, 3 ml of SOC medium (20mM glucose pH 7.0, 2% bacto-tryptone, 0.5% yeast extract,

0.05% $NaCl_2$, 2.5mM KCl) was added and the culture was shaken at 220 rpm for 1 hr at 37°C after which 10 ml of SB (super broth; 30 g of tryptone, 20 g of yeast extract, and 10 g of Mops per liter, pH 7) containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added. At this point, samples (20, 1, and 0.1 μl) were withdrawn for plating to determine the library size. Typically the library had about 10^7 members. The culture was grown for an additional hour at 37°C while shaking at 300 rpm. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and was grown overnight. Phagemid DNA containing the heavy-chain library was prepared from this overnight culture. To determine the insert frequency of this ligation, 10 colonies from the plates used to titer the library were picked and grown. DNA was prepared and then digested with Xho I and Spe I.

For the cloning of the light chain, phagemid DNA (pcomb3) (10 μg) was digested as described above except that the restriction enzymes Sac I and Xba I were used. The resulting linearized vector was treated with phosphatase and purified by agarose gel electrophoresis. The desired fragment, 4.7 kilobases long, was excised from the gel. Ligation of this vector with prepared light-chain PCR DNA proceeded as described above for the heavy chain. After transformation, 3 ml of SOC medium was added and the culture was shaken at 220 rpm for 1 hour at 37°C. Then 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10 μg/ml) was added (samples were removed for titering as described above for the heavy-chain cloning) and the culture was shaken at 300 rpm for an additional hour. This culture was added to 100 ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and then shaken for 1 hr. Helper phage VCS-M13 (10^{12} plaque-forming units) was added and the culture was shaken for an additional 2 hours. After this time, kanamycin (70 μg/ml) was added and the culture was incubated at 37°C overnight. The supernatant was cleared by centrifugation (4000 rpm for 15 minutes in a JA-

10 rotor) at 4°C. Phage were precipitated by addition of 4% (wt/vol) polyethylene glycol 8000 and 3% (wt/vol) NaCl followed by incubation on ice for 30 minutes and centrifugation. Phage pellets were resuspended in 2 ml of phosphate-buffered saline (PBS: 50 mM phosphate, pH 7.2/150 mM NaCl) and microcentrifuged for 3 minutes to pellet debris. Supernatants were transferred to fresh tubes and stored at -20°C.

Example 7: Titering of Colony-Forming Units

Phagemids that have been packaged into virions are capable of infecting male *E. coli* to form colonies on selective plates. Phage (packaged phagemid) was diluted in SB (dilutions: 10^{-3}, 10^{-6}, and 10^{-8}) and 1 μl was used to infect 50 μl of fresh *E. coli* XL1-Blue culture (OD_{600}=1) grown in SB containing tetracycline (10 μg/ml). Phage and cells were incubated at room temperature for 15 minutes and then directly plated on LB/carbenicillin plates.

Example 8: Panning of the Combinatorial Library to Select Antigen Binders

Four wells of a microtiter plate (Costar 3690) were coated overnight at 4°C with 25 μl of recombinantly produced rev or tat protein (40 μg/ml in 0.1 M bicarbonate buffer, pH 8.6). The wells were washed twice with water and blocked by completely filling the well with 1% (wt/vol) bovine serum albumin (BSA) in PBS and incubating the plate at 37°C for 1 hour. Blocking solution was shaken out, 50 μl of the phage library (typically 10^{11} colony-forming units) was added to each well, and the plate was incubated at 37°C for 2 hours. Phage were removed and the plate was washed once with water. Each well was then washed 10 times with 50 mM Tris-HCl, pH 7.5/150 mM NaCl/0.5% Tween 20 over a period of 1 hour at room temperature. The plate was washed once more with distilled water and adherent phage were eluted by the addition of 50 μl of elution buffer (0.1 M HCl, adjusted to pH 2.2 with solid glycine and containing 0.1% BSA) to each well and incubation at room temperature for 10 minutes. The elution buffer was pipetted up and down several times, removed, and neutralized with 3 μl of 2 M Tris base per 50

μl of elution buffer used. Eluted phage were used to infect 2 ml of fresh *E. coli* XL1-Blue cells (OD_{600}=1) for 15 minutes at room temperature after which 10 ml of SB containing carbenicillin (20 μg/ml) and tetracycline (10μg/ml) was added. Samples (20, 1, and 0.1 μl) were removed for plating to determine the number of phage (packaged phagemids) that were eluted from the plate. The culture was shaken for 1 hour at 37°C and then added to 100ml of SB containing carbenicillin (50 μg/ml) and tetracycline (10 μg/ml) and shaken for 1 hour. Helper phage VCS-M13 (10^{12} plaque-forming units) were added and the culture shaken for an additional 2 hours. Then kanamycin (70 μg/ml) was added and the culture was incubated at 37°C overnight. Phage preparation and further panning were repeated four times as described above.

Example 9: Preparation of Soluble Fab Fragments

Phagemid DNA from positive clones was isolated and digested with *Spe I* and *Nhe I*. Digestion with these enzymes produces compatible cohesive ends. The 4.7-kilobase DNA fragment lacking the gene III (cap protein) portion was gel-purified (0.6% agarose) and self-ligated.

Transformation of *E. coli* XL1-Blue afforded the isolation of recombinants lacking the gene III (cap protein) fragment. Clones were examined for removal of the gene III fragment by *Xho I/Xba I* digestion, which yielded a 1.6-kilobase fragment. Clones were grown in 15 ml of SB containing carbenicillin (50μg/ml) and 20 mM $MgCl_2$ at 37°C until OD_{600} of 0.2 was achieved.

Isopropyl β-D-thiogalactopyranoside (1mM) was added and the culture was incubated overnight at 37°C. Cells were pelleted by centrifugation at 4000 rpm for 15 minutes in a JA-10 rotor (Beckman J2-21) at 4°C. Cells were resuspended in 3 ml of PBS containing 0.2 mM phenylmethylsulfonyl fluoride and lysed by sonication on ice (2-4 min, 50% duty). The debris was pelleted by centrifugation at 14,000 rpm in a JA-20 rotor at 4°C for 15

minutes. The supernatant was used directly for ELISA analysis and was stored at -20°C.

Example 10: ELISA Analysis of Human anti-rev and anti-tat Fab Supernatants

ELISA wells were coated with rev and tat proteins exactly as above, washed five times with water, blocked in 100 µl of 1% BSA/PBS for 1 hour at 37°C, and then incubated with 25 µl Fab supernatants for 1 hour at 37°C. After 10 washes with water, 25 µl of a 1:1000 dilution of alkaline phosphatase-conjugated goat anti-human IgG $F(ab')_2$ (Pierce) was added and incubated for 1 hour at 37°C. Following 10 washes with water, 50 µl of *p*-nitrophenyl phosphate substrate was added and color development was monitored at 405 nm. Positive clones gave A_{405} values >1 (mostly >1.5) after 10 minutes, whereas negative clones gave values of 0.1-0.2.

Three Fab producing clones were isolated against HIV-1 rev and 4 Fab producing clones were isolated against HIV-1 tat. Results are shown in Table 1.

TABLE 1

Properties of Human Monoclonal Fab Derived from an Asymptomatic, 10 yr HIV-1 Positive Donor

ANTIGEN	CLONE	BINDING CONSTANT	YIELD/ LITER (µg)	TITER
HIV-1 rev	rev 9(Fd)	8×10^{-7}M	20	1/1
	rev 9/12LC			
	rev 9/16LC	6×10^{-8}M	12	1/4
HIV-1 rev	rev 16	6×10^{-7}M	67	1/2
HIV-1 rev	rev 20	6×10^{-7}M	39	1/2
HIV-1 tat	tat 31	4.2×10^{-7}M	94	1/8
HIV-1 tat	tat 16	1.7×10^{-6}M	66	1/1
HIV-1 tat	tat 104	3.2×10^{-7}M	84	1/8
HIV-1 tat	tat 107	3.0×10^{-7}M	12	1/1

Example 11: Sequencing

Nucleic acid sequencing was carried out on double-stranded DNA with Sequenase 1.0 (United States Biochemical). Amino acid sequences were determined and are set forth in Table 2.

TABLE 2
HUMAN ANTI-HIV Fab AMINO ACID SEQUENCES

CLONE	SEQ ID NO.	FR1	CDR1	FR2
Heavy Chain VH Sequences				
rev9(VH3)	6	LLESGGGVVQPGRSLRLSCAASGFIFS	TYGIY	WVPQAPGKGLEWVA
rev16(VH3)	7	LLESGGGLAQPGGSLRLSCAASGFTFS	SYEMN	WVRQPPGKGLEWVS
rev20(VH3)	8	LLESGGGLAQTGGSLRLSCAASGFTFS	SYEMN	WVRQPPGKGLEWVS
tat104(VH3)	9	LLESGGGGVVQPGGSLRLSCEASGFSLI	NTAMH	WVRQAPXKGPEWVS
Light Chain VL Sequences				
rev16/20 (VL5-FR1, CDR1, CL)	10	AELQPPSVSAAPGQKVIISC	SGSSSHT/ /GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAXSS	
tat16(VL1)	11	AELQPPSVSAAPGQKVTISC	SGSTSNIGNRHVS	WYQQLPGTXPKLLIY
tat31(VL1)	12	AELQPPSVSAAPGQSVTISC	SGSSSNIGNYNVX	WYQQTPGSAPKTLIY
tat104(VL4)	13	GELQDPVVSVALGQTVRMTC	QGDSLRYHYAN	WYQQKPGQAPILVIK
tat107(VL4)	14	AELQDPVVSVALGQTVRITC	QGDSLRXYHAN	WYQQKPGKAPIFVIY
Light Chain CL Sequences				
rev16/20	15	/GQPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKAGVETTTPSKQSNNKYAXSSYLSLTPEQWKSHKSYXCQVTHEGSTVEKTVXPTECS		
tat16	16	QPKXAPSVTLFPPSSEELQANKXTLVCLISDFFPGXXXVXWKXDSXPXKGGVETTXPP		
tat31	17	QPKXAPSVTLFPPSSEELQANKXTLVCLISDFYPGAXTVXWKADSSPVKAGVENTTPSIXMQQQVSGPGGI		
tat104	18	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVTVAWKADSSPVKGGVETTTPSNQSNNKFAASRYLSLTPEQWKSHRSYSCQV		
tat107	19	QPKAAPSVTLFPPSSEELQANKATLVCLISDFYPGAVXVAWKADSSPVKVGVXXTTPSXHXINMFAGSXYLSLTPEQWXSHRKLQLPGQRRMXGAPXR		

HUMAN ANTI-HIV Fab AMINO ACID SEQUENCES				
CLONE	**CDR2**	**FR3**	**CDR3**	**FR4**
Heavy Chain VH Sequences				
rev9(VH3)	IISHDGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTXVYYCAR	EGVHKXFDH	WGQGTLVTVSSASTKGPSV
rev16(VH3)	YISSGSDTIYYADSVKG	RFIISRDNAKNTLYLQMNNLRGEDTAVY' CAR	DPRRWTQLWIPPDY	WGQGTLVSVSSASTKGPSV
rev20(VH3)	YISSGSDTIYYADSVKG	RFIISRDNAKNTLYLQMNNLRGEDTAVYYCAR	DPRRWTQLWIPPDY	WGQGTLVSVSSASTKGPSV
tat104(VH3)	VSSYDGREKYYTDSVKG	RFSISRDDSTNMLYLQMNSVKIDDTAVYYCAR	TNRAYCSGVRCHDGLDV	WGQGTMVTVSSASTKGPSV
Light Chain VL Sequences				
rev16/20 (VL5-FR1, CDR1, CL)	YLSLTPEQWKSHKSYXCQVTHEGSTVEKTVXPTECS			
tat16(VL1)	ENNIRPS	GIPDRFSASKSGTSATLDITGLQTGDEADYYC	GTWDSSLSTGHWV	FGGGTKLTVLS
tat31(VL1)	DTHKRPS	GISERFSGSKSGTSATLGITGLQTGDEADYYC	GTWDTSLNSAL	FGGGTKLTXLG
tat104(VL4)	SKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	DSRDTSGNHPRVL	FGGGTKLTVLG
tat107(VL4)	GKNNRPS	GIPDRFSGSSSGNTASLTITGAQAEDEADYYC	NSRDSSSNHVV	FGGGTKLTVLG
Light Chain CL Sequences				
rev16/20				
tat16				
tat31				
tat104				
tat107				

Example 12: Epitope Mapping

ELISA assays were performed as described above using defined peptides of the tat and rev proteins set forth in Tables 2 and 3, respectively. The anti-tat Fab bound to the cysteine rich tat functional domain as shown in Figure 12. Reduction of the antibody reduced binding of the Fab to the functional domain as shown in Figure 13.

Binding of anti-rev Fd and Fab is shown in Figure 14. The anti-rev Fd rev9 bound to the sequence immediately adjacent to the basic nucleolar localization domain. Anti-rev Fab rev16 and rev20 were found to be identical and binding was evident to the region immediately adjacent to the activation domain.

Examples 13 to 23, provided below, comprise a description of experiments establishing that intracellular immunization of cells can be successfully accomplished using retroviral vectors encoding sFvs directed against essential HIV-1 genes. In addition, these data provide further support for the feasibility of intracellular immunization as a viable means of treating HIV infection. The data describe the development and characterization of amphotropic murine retroviral shuttle vectors which express intracellularly anti-rev sFv. Using these vectors, human T-lymphotrophic cell lines were transduced with anti-rev sFV and were thereby intracellularly immunized against infection with HIV. Potent inhibition of HIV-1 replication was evident in cells so immunized. This was the case both in cell clones and in mixed cell populations. Further, peripheral blood mononuclear cells (PBMC), obtained from HIV seronegative individuals could be efficiently transduced with the expression vectors of the invention.

Example 13: Cells and viruses used to generate and test retroviral vectors encoding sFv

The HIV-1 viral strains utilized in the experiments described herein include HIV-1 strain NL4-3 (Adachi et al., J. Virol. 59:284-291, 1986) and HIV-1 strain

HXB2 (Ratner et al., Nature 313:277-284, 1985). NL4-3 (in which all viral open reading frames are intact and is highly cytopathic for T-lymphocytes) and HXB2 were produced from transfected Cos cells and propagated in the T-lymphocytic cell-line, CEM. Viral stocks were assayed for their infectious titers on both CEM and H9 cells (Aldovini et al., In: Techniques in HIV Research, Stockton Press, NY, 1990). An HIV-1 viral construct (HIV-CAT), containing the CAT gene inserted into the *nef* open reading frame (Malim et al., J. Exp. Med. 176:1197-1201, 1992), was also used in some experiments as indicated herein.

CEM, H9, and Sup-T1 cells are CD4-positive human T-lymphocytic cell lines, which are highly susceptible to infection with HIV-1 (Aldovini et al., In: Techniques in HIV Research, Stockton Press, NY, 1990). These cells were grown in RPMI-1640 medium, supplemented with 10% fetal calf serum (FCS) (Gibco-BRL). Retroviral packaging cells, PA317 (Miller et al., Biotechniques 7:980-990, 1989), were maintained in Dulbecco's modified Eagle's medium (DMEM) in the presence of 10% FCS. Human PBMC were prepared via Ficoll-Hypaque centrifugation of blood from HIV-1-seronegative individuals. The PBMC were stimulated with phytohemagglutinin (PHA) (5 μg/ml - Sigma) and interleukin-II (IL-2) (50 U/ml - Sigma) for 3 days, following which the PBMC were maintained in RPMI-1640 medium, supplemented with 20% FCS and IL-2 (10 U/ml).

Example 14: Construction of retroviral vectors and transduction of target cells

Two murine leukemia virus (MLV)-based retroviral shuttle vectors, pLXSN and PSLXCMV, were used in the experiments described herein. These expression vectors contain the bacterial neomycin-resistance gene (neo) (Miller et al., Biotechniques 7:980-990, 1989). Genes of interest were inserted in the polylinker regions of these vectors (see Figure 15). β-Galactosidase (β-Gal) and CAT-expression retroviral vectors, PLXSP-Gal and PLXSCAT, were constructed as described herein. PSLXCMV-CAT was constructed by

inserting a 726 bp Hind III (blunt)-Bam HI fragment, containing the CAT gene into pSLXCW via Hpa I-Bgl II sites. The anti-rev sFv (D8) moiety used in these studies, constructed from the V_l and V_h cDNA obtained from a murine hybridoma, is also described herein. For anti-rev (D8) sFv expression, a 869 bp Xho I-Bam HI sFv-containing fragment was inserted into the Xho I-Bam HI sites of pLSXN, to form pLSXN-D8-SFv. In order to insert the anti-rev sFv into the pSLXCMV vector, a Xho I (blunt)-Bam HI sFv-containing fragment was ligated into a Hpa I-Bgl II treated pSLXCMV vector, to form pSLXCW-D8-sFv.

Helper-free, recombinant MLV viral stocks were produced by transient transfection of retroviral vector plasmids into PA317 cells, which were used in all experiments to produce the various retroviral shuttle vector virions. Briefly, 5 μg of purified plasmids were transfected into 1 X 10^6 PA317 cells/100 mm dish, using a standard lipofectamine reagent procedure (Gibco-BRL). Five hours later, 10 ml of pre-warmed DMEM supplemented with 20% FCS was added to the cells. At 48 hours post-transfection, the supernatants were harvested by centrifugation at 1500 g for 5 minutes. For transduction of CEM and Sup-T1 cells, 5 ml of the transfected PA317 cell supernatants were used to infect 1-2 X 10^6 target cells with polybrene (8 μg/ml) overnight. Cells were then washed with serum free media and maintained in G418 (400 μg/ml) for 2 days. Clonal cell lines were isolated by G418 selection and limiting dilution. In addition, mixed cellular populations were isolated by continuous culture in G418 (1 mg/ml)-containing medium for 2 weeks.

For human PBMC, after 3 days of PHA/IL-2 stimulation (PHA: 5 μg/ml, IL-2: 50 U/ml), 1 X 10^6 cells were cultured with 10 ml of transfected PA317 supernatant and incubated for 3 days with daily replacement of fresh packaging cell line supernatant. The transduced PBMC were then challenged with HIV-1.

Example 15: Cat Assays

CAT assays were performed as described (Gorman et al., Mol. Cell. Biol. 2:1044-1051, 1982; Ausebel et al., Current Protocols in Molecular Biology, Volume 1, John Wiley and Sons, NY). Briefly, 1 X 10^7 cells were transduced using the CAT-expression retroviral vectors, and were harvested by centrifugation. The pelleted cells were lysed in 0.9 ml CAT lysis buffer (Promega, Inc.) and approximately 200 μl of supernatants, normalized for protein content, were used in standard CAT assays. Percent conversion of chloramphenicol was assessed using a phosphor imager (Molecular Dynamics, Inc.).

Example 16: sFv DNA-PCR and Immunostaining for sFv Expression

To measure intracellular sFv gene expression and protein localization several complementary tests were performed. First, to perform sFv-DNA-PCR, twenty clones from stable G418-selected and transduced CEM and Sup-T1 cells were maintained in G418-free medium for two months. Samples of 0.5 X 10^5 cells were pelleted and washed with phosphate-buffered saline (PBS). The cells were resuspended in 200 μl H_20 and the samples were boiled for 3-5 minutes and then immediately cooled on ice. Five microliters of supernatant from each sample was used for PCR amplification of the sFv gene in a 20 μl reaction, using specific oligonucleotide primers: EAR-5: 5'-CCAGATCTGATGTGCAGCTGGTGGAGTC-3'and EAR-6: 5'-TTGGATCCTCAGGATAGACGGGTGGGGGTG-3'. The PCR cycles were: 94°C for 1 minute and 20 seconds, 50°C for 2 minutes and 72°C for 1 minute and 30 seconds, for 35 cycles with a final extension for 10 minutes at 72°C. PCR amplification products were resolved on 1.5% agarose gels.

Intracellular sFv gene expression and protein localization was detected by indirect immunofluorescence assays (Bagasra et al., Proc. Natl. Acad. Sci. USA 89:6285-6289, 1992). Briefly, retroviral vector-transduced cloned cells were cultured on multichambered glass slides overnight. After removing the culture media, the cells were

fixed in 10% methanol and 90% ethanol at -20°C for 2 hours. Cells were then washed twice with PBS and blocked with 5% normal goat serum for 1 hour at 37°C. Cells were further overlayed with 200 μl of polyclonal rabbit anti-mouse IgG (Fab-specific) (Sigma), for 2 hours at 37°C. After washing five times with PBS, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG for 1 hour. Following an additional five washes in PBS, the cells were analyzed by epifluorescence microscopy.

Example 17: Infection of cells with HIV-1

Viral stocks of the HIV-1 strains, HXB2 and NL4-3 were used in these studies. G418-selected CEM and Sup-T1 cells were first maintained in G418-free medium for two weeks prior to HIV-1 infection. Also prior to infection, cell-surface CD4 antigen expression was analyzed by flow cytometry (see below). Parental CEM and Sup-T1 cells and CAT-transduced and sFv-transduced CEM and Sup-T1 cells were incubated with infectious HIV-1 strains HXB2 and NL4-3 at various input multiplicities of infection for 2 hours following which the cells were washed four times in pre-warmed, serum-free media. Human PBMC were challenged with HIV-1 strain NL4-3 using mois of 0.24 and 0.06. Cells were cultured at an initial concentration of 5 X 10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS for CEM and Sup-T1 cells, and 20% FCS for human PBMC. On day three post-infection, the cells were split at a ratio of 1:4 to maintain a cell concentration of approximately 5 X 10^5/ml. The culture supernatants were collected every three days after infection and the level of HIV-1 p24 antigen in the supernatants was determined by an HIV-1 antigen capture ELISA. Cell viability was monitored by trypan blue exclusion staining.

Example 18: Fluorescence-Activated Cell Sorting (FACS) Analyses for Surface CD4 Antigen

For FACS analysis, cells were treated with an anti-CD4a FITC-conjugated antibody (Sigma). Cells were then

analyzed on a FACScan instrument (Becton-Dickinson, Inc.), as described (Bagasra et al., Proc. Natl. Acad. Sci. USA 89:6285-6289, 1992).

Example 19: Gene Expression Using Retroviral Shuttle Vectors in Human T-Lymphocytic Cell Lines and Human PBMC

An MLV-based retroviral system was used in this study (Figure 15). To determine whether these retroviral vectors are capable of measurable levels of gene expression of a given gene inserted therein, the expression of CAT and β-Gal inserted therein was first evaluated. CAT expression by these vectors was assessed in both human T lymphocyte cell lines and in PBMC (Figures 16 and 17). In addition, β-gal expression was assessed to provide an estimate of the transduction efficiency of these retroviral shuttle systems in these cell types. From the data presented in Figures 2 and 3 it is evident that the vectors pLSXN and pSLXCMV, maintain excellent levels of CAT expression over a period of 2 to 6 months in stable CEM and Sup-T1 cell-lines (Figures 16 and 17A). In most experiments, the transduction efficiency of 1 X 10^6 Sup-T1 or CEM cells, using 5 ml of fresh packaging cell supernatant, was greater than 60% (as assessed by β-gal staining). Because variations in the levels of gene expression levels have been observed following G418 selection in single cell clonal populations, forty CAT-vector transduced clones were isolated and analyzed. Each of these clones was tested for CAT expression following maintenance in culture for two months. The data presented in Figure 16 establish that there was relatively little variation in CAT expression in most of these clones. Interestingly, as can be seen in Figure 17A (lanes 3 and 4), pLSXN, in which the MLV LTR directly drives CAT expression, exhibited greater levels of CAT activity in Sup-T1 cells as compared with pSLXCMV-transduced Sup-T1 cells. Further, pLSXN exhibited greater levels of CAT activity in Sup-T1 cells compared with CEM cells which were transduced with this vector (Figure 17A, lanes 3 and 5).

The vector pSLXCMV, in which CAT gene expression is driven by an internal cytomegalovirus (CMV) promoter, yielded higher levels of CAT in human PBMC compared with PBMC transduced with pLSXN. These data correlate with those obtained in the HIV-1 challenge experiments described below.

To assess the stability and expression of the inserted sFv gene in human T lymphocytes, G418-selected, sFv-transduced CEM and Sup-T1 cellular clones were maintained in G418-free media for two months and then assayed for the presence and expression of sFv using immunofluorescence and a DNA- and reverse transcriptase (RT)-PCR assay. As shown in Figure 18A, most of the cell clones stably maintained the sFv gene. Of the 40 CEM cell clones which were tested, only one clone did not contain sFv DNA (Figure 18A, lane 3). Expression of sFv mRNA was confirmed by RT-PCR, and cytoplasmic localization of the sFv protein was demonstrated by specific immunostaining of the anti-rev sFv in both T lymphocytes and transduced PBMC (Figure 18B).

Example 20: Intracellular Anti-rev SFv Potently Alters Expression of HIV-1 in Human T Lymphocytes

Cellular clones and mixed cellular populations of CEM and Sup-T1 cells transduced with either anti-rev sFv or control CAT-expressing retroviral vectors were analyzed for their ability to support the replication of HIV-1. The mixed cell populations were infected with HIV_{HXB2} at an moi of 0.24 or with HIV_{NL4-3} at an moi of 0.024. Replication of either strain of HIV-1 in the cultures was assessed using a p24 antigen ELISA (Figure 19). Parental non-transduced CEM and Sup-T1 cells and CAT-transduced cells supported vigorous replication of HIV-1, as shown by the initial increase in HIV-1 p24 antigen which peaked at approximately 12-15 days post-infection. Subsequent decreases in HIV-1 p24 antigen production in control cells is a reflection of HIV-1 induced cell killing in this population. As demonstrated by the data shown in Figure 19, in these relatively high moi input experiments, HIV-1 replication was strongly inhibited in the

mixed populations of sFv-transduced Sup-T1 and CEM cells. In experiments in which sFv-transduced cellular clones of CEM and Sup-T1 cells were compared with parental non-transduced and CAT-transduced cells, four out of five clones harboring an intact sFv provirus and expressing sFv virtually ablated HIV-1 replication as evaluated over two months after initial HIV-1 infection (Figure 20). In addition, anti-rev sFv-transduced T-lymphoid cells were observed to be refractory to HIV-1 induced syncytia formation induced by HIV-1 infection (Figure 21).

Example 21: Inhibition of HIV-1 Replication by Anti-Rev SFv in Human PBMC

To evaluate the potential protective effects of intracellular anti-rev sFv expression against HIV-1 infection in primary blood mononuclear cells, stimulated human PBMC transduced with retroviral vectors expressing either anti-rev sFv or CAT, were challenged with relatively high mois of HIV-1 (0.24 and 0.06). The data shown in Figure 22 illustrates the dramatic inhibition of HIV-1 production in cells expressing anti-rev sFv (greater than 98% inhibition in several experiments). The vector pSLXCMV-D8-sFv may be an even more potent inhibitor of HIV-1 at the higher moi in PBMCs compared with the vector pLXSN-D8-sFv. These challenge experiments were performed using fresh PBMC from several different HIV-1-seronegative donors. In each instance, the results obtained were similar. Thus, potent inhibition of HIV-1 replication by an intracellularly expressed sFv which has as its target a retroviral regulatory protein, is now demonstrated in human PBMC.

Example 22: Inhibition of HIV-1 Replication in sFv-Transduced Cells is Secondary to a Specific Block in Rev Function

Because each of the cells which were used were selected in G418, it was necessary to ensure that the resistance of sFv-modified cells to HIV-1 infection was the result of inhibition of rev function and was not due to some other nonspecific G418 induced effect. Rev enhances the

accumulation of unspliced HIV-1 RNA in the cytoplasm of HIV-1-infected cells (Cullen, J. Virol. 65:1053-1056, 1991). Consequently, in cells expressing anti-rev sFv, if rev is specifically inhibited, the early stages of HIV-1 infection should not be affected but instead HIV-1 replication will be inhibited at a later stage of the virus life-cycle.

To evaluate these parameters, the levels of surface CD4 antigen were compared both before and after G418 selection by staining cells with a specific anti-CD4 antibody. As shown in Figure 23A, when cells were selected for two weeks in G418 and then cultured in G418-free medium for two additional weeks, cell-surface CD4 antigen expression was not affected in transduced versus non-transduced CEM and Sup-T1 cells. In addition, no difference in the pattern of CD4 antigen expression was observed in transduced versus non-transduced PBMC (Figure 23A).

In a second series of experiments, sFv-transduced cells were infected with a genetically-modified HIV-1 strain (Malim et al., J.Exp. Med. 176:1197-1201, 1992). This HIV-1 clone HIV-CAT) encodes the CAT gene within the *nef* open reading frame and, thus, CAT is expressed as an early gene product from multiply-spliced viral RNA through a rev-independent mechanism in cells infected with this virus. HIV-CAT expression in different clonal and mixed cell populations clearly establish that the transduced cells are equally sensitive to HIV-1 infection at the early stages of virus replication (Figure 23B). These early stages, from the point of virion binding through early viral mRNA expression, are not affected by rev function (Pomerantz et al., J. Virol. 66:1809-1813, 1992). Thus, the high levels of CAT expression in cells infected with HIV-CAT in anti-rev sFv-transduced cells demonstrate that the inhibitory effects of sFv on HIV-1 replication in T-lymphocytes is specific for the later stages of the virus life-cycle.

Example 23: Inhibition of replication of primary isolates of HIV-1 in anti-rev transduced human PBMC

Human PBMC were stimulated by treatment with 5 μl/ml of PHA and 50 μl/ml of IL-2 for three days. Samples of 1 X 10^6 cells so stimulated were cultured in the presence of 10 ml of transfected packaging cell line PA317 supernatant for 3 days with daily replacement of the supernatant. The cells were transduced with the vectors LXSNSF8 and LXSNCAT as described herein. The transduced PBMC which were cultured in IL-2 alone were then challenged with one of two clinical isolates of HIV-1. These isolates, HIV-1 strain NSI #89000641 and HIV-1 strain SI #9200611, were isolated from the PBMC of two infected children. Both strains are resistant to AZT; further, strain NSI #89000641 does not induce syncytium formation whereas strain SI #9200611 induces syncytium formation in infected cells.

For infection of PBMCs, 100 pg of HIV-1 p24 equivalents of virus was mixed with the transduced PBMC for 2 hours at 37°C following which the cells were washed five times in 10 ml of prewarmed serum free medium. The cells were then resuspended in 2 ml of culture medium containing IL-2 following which they were maintained at a density of 0.2 to 0.5 X 10^6 cells/ml by splitting the cells at regular intervals. Samples were obtained on the days indicated in Figure 24 and the amount of p24 antigen production was measured. The data presented in Figure 24 establish that expression of anti-rev sFv in human PBMC confers upon these cells the ability to inhibit the replication of clinical isolates of HIV-1.

In summary, the data presented herein establish that anti-rev sFv-transduced human T lymphoid cells or human PBMC inhibit HIV-1 replication. The replication of both laboratory clones of virus and of fresh clinical isolates is inhibited by anti-rev sFvs. Further, the inhibition of HIV-1 replication is dependent on specific alterations in rev function.

Example 24, provided below, establishes that yet other sFvs (in addition to HIV-1 rev and tat) directed against other HIV-1 genes are also useful as intracellular

immunizing agents to effect inhibition of HIV-1 replication.

Exampl 24: Cloning and characterization of sFvs dir cted against HIV-1 integrase (IN)

The HIV-1 integrase (IN) gene plays an essential role in the virus replication cycle in that it mediates integration of the viral genome into the host cell genome. The 288 amino acid integrase protein has three domains. The amino terminal domain has a zinc finger-like motif of two histidines followed by two cysteines (HHCC domain). The central domain comprises the catalytic center containing a highly conserved D,D-35-E motif. The carboxyl terminal domain binds to DNA and is required for efficient 3' processing and DNA strand transfer. In the experiments described herein, anti-IN monoclonal antibodies have been generated which are specific for each of the domains within the IN protein. A diagram of the IN gene is presented in Figure 25. The relative positions of the domains of the protein are also shown as are the relative positions on the molecule to which each monoclonal antibody is directed. Also shown in this figure is the relative binding affinities of each of the monoclonal antibodies to the appropriate domains on IN and the relative ability of each of these antibodies to inhibit IN function is also shown.

Specifically, to generate anti-IN sFvs, five hybridoma cells lines were used expressing monoclonal antibodies directed against all three domains on the IN protein. Cloning of the heavy (Vh) and light (Vl) chain variable genes from the hybridoma cell lines was accomplished using the Novagen Ig prime kit. Each Vh domain was cloned by isolating cellular RNA and synthesizing the first strand of DNA using a mouse 3'Vh primer. PCR amplification of cDNA using 3' and 5' Vh primers generated a 450 bp DNA fragment which was then cloned into the T7 Blue (R) vector in order that it be sequenced. Each Vl domain was cloned in a similar manner with the following exceptions: Since the parent hybridoma cell line sp2/0 contains an aberrant variable kappa chain (abVk), reduction

of the level of the abVk mRNA level was accomplished by cocultivation of the hybridoma cell lines with pA317 cells transiently expressing MLV which, as disclosed herein, encodes an abVk specific ribozyme. Vl first strand cDNA was synthesized using a 3' Vl primer and isolated ribozyme treated hybridoma RNA. Light chain cDNA was amplified by PCR using 3' Vl and 5' Vl primers. Progeny clones were screened by PCR for the presence of the abVk sequence using specific abVk primers (200 bp product). Clones which were negative for abVk were screened by PCR for Vl sequences using T7 and U19 pT vector primers (Novagene) (500 bp product). All of the Vh and Vl clones which were obtained were sequenced in both orientations to identify novel anti-IN antibody sequences.

Anti-IN sFv molecules were generated by simply replacing rev in the anti-rev sFv constructs disclosed above with the cloned anti-IN sequences. PCR amplification was used to add convenient restriction enzyme sites which are either 5' or 3' to each Vl and Vh domain. Each of these anti-IN sFv molecules was sequenced in order to insure that they did not contain additional mutations resulting from the cloning procedure. Each of the anti-IN sFv was cloned into the following expression vectors: pSLX-CMV and pSLXN, retroviral expression vectors; pET-19b, a bacterial expression vector and pSFV1, a high copy expression vector. A diagram of each of these vectors depicting the relative position of the inserted IN gene is presented in Figure 26.

The nucleotide and corresponding amino acid sequence of each of the IN-specific antibody molecules is presented in Figure 27.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Thomas Jefferson University

(ii) TITLE OF INVENTION: Intracellular Immunization

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Woodcock Washburn Kurtz Mackiewicz and Norris
(B) STREET: One Liberty Place - 46th Floor
(C) CITY: Philadelphia
(D) STATE: PA
(E) COUNTRY: U.S.A.
(F) ZIP: 19103

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette, 3.5 in., 1.44 Mb storage
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: WordPerfect 5.1

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: herewith - 23 May 1996
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Ralph, Rebecca L.
(B) REGISTRATION NUMBER: 35,152
(C) REFERENCE/DOCKET NUMBER: TJU-1903

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 215-546-8396
(B) TELEFAX: 215-568-3439

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser Gly Gly Gly Gly Ser
1 5 10 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 861 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGGCCATC ATCATCATCA TCATCATCAT CATCATAGCA GCGGCCATAT CGACGACGAC 60

GACAACCATA TGTTGGTGCT GACGTTCTGG ATTCCTGCTT CCAGCAGTGA TGTTGTGATG 120

GCCCAAACTC CACTCTCCCT GCCTGTCAGT CTTGGACATC AAGCCTCCAT CTCTTGCATA 180

TCTAGTCAGA GCCTTGTACA CAGTAATGGA AACACCTATT TACATTGGTA CCTGCAGAAG 240

CCAGGCCAGT CTCCAAAGCT CCTGATCTAC AAAGCTTCCA ACCGATTTTC TGGGGTCCCA 300

GACAGGTTCA GTGGCAGTGG ATCAGGGACA GATTTCACAC TCAAGATCAG CAGAGTGGAG 360

GCTGAGGATC TCCCAGTTTA TTTCTGCTCT CAAAGTACAC ATTTTCCGTG GACGTTCGGT 420

GGAGGCACCA AGCTGGAAAT CAAACGGGCT GATGGGCCCG GTGGGGGCGG TTCGGGTGGC 480

GGGGGCTCGG GCGGGGGTGG CTCAGAGCTC GGCAGATCTG ATGTGCAGCT GGTGGACTCT 540

GGGGGAGGGT TAGTGCAGCC TGGAGGGTCC CGGAAACTCT CCTGTGCAGG CTCTGGATTC 600

ACTTTGACTA GGTTTGGAAT GCACTGGGTT CGGCAGGCTC CAGAGAAGGG GCTGGACTGG 660

GTCGCATACA TTAGTAGTGG GAGTAGTACC CTCCACTATG CAGACACAGT GAAGGGCCGA 720

TTCACCATCT CCAGACACAA TCCCAAGAAC ACCCTGTTCC TGCAAATGAA ACTACCCTCA 780

CTATGCTATG CACTACTGGG GTCAAGGAAC CTCAGTCACC GTCTCCTCAG CCAAAACGAC 840

ACCCCCACCC GTCTATCCTG A 861

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Met Arg Ala Lys Leu Leu Gly Ile Val Leu Thr Thr
1 5 10

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Gly Arg Lys Lys Arg Arg Gln Arg Arg Arg Ala His Gln Asn
1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 51 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GGCAGGAAGA AGCGGAGACA GCGACGAAGA GCTCATCAGA ACAGTCAGAC T
51

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 123 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Leu Leu Glu Ser Gly Gly Gly Val Val Gln Pro Gly Arg Ser Leu Arg
1 5 10 15

Leu Ser Cys Ala Ala Ser Gly Phe Ile Phe Ser Thr Tyr Gly Ile Tyr
20 25 30

Trp Val Pro Gln Ala Pro Gly Lys Gly Leu Glu Trp Val Ala Ile Ile
35 40 45

Ser His Asp Gly Ser Asn Lys Tyr Tyr Ala Asp Ser Val Lys Gly Arg
50 55 60

Phe Thr Ile Ser Arg Asp Asn Ser Lys Asn Thr Leu Tyr Leu Gln Met
65 70 75 80

Asn Ser Leu Arg Ala Glu Asp Thr Xaa Val Tyr Tyr Cys Ala Arg Glu
85 90 95

Gly Val His Lys Xaa Phe Asp His Trp Gly Gln Gly Thr Leu Val Thr
100 105 110

Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Leu Leu Glu Ser Gly Gly Gly Leu Ala Gln Pro Gly Gly Ser Leu Arg
1 5 10 15

Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Glu Met Asn
20 25 30

Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Val Ser Tyr Ile
35 40 45

Ser Ser Gly Ser Asp Thr Ile Tyr Tyr Ala Asp Ser Val Lys Gly Arg
50 55 60

Phe Ile Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met
65 70 75 80

Asn Asn Leu Arg Gly Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp
85 90 95

Pro Arg Arg Trp Thr Gln Leu Trp Ile Pro Pro Asp Tyr Trp Gly Gln
100 105 110

Gly Thr Leu Val Ser Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120 125

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 128 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Leu Leu Glu Ser Gly Gly Gly Leu Ala Gln Thr Gly Gly Ser Leu Arg
1 5 10 15

Leu Ser Cys Ala Ala Ser Gly Phe Thr Phe Ser Ser Tyr Glu Met Asn
20 25 30

Trp Val Arg Gln Pro Pro Gly Lys Gly Leu Glu Trp Val Ser Tyr Ile
35 40 45

Ser Ser Gly Ser Asp Thr Ile Tyr Tyr Ala Asp Ser Val Lys Gly Arg
50 55 60

Phe Ile Ile Ser Arg Asp Asn Ala Lys Asn Thr Leu Tyr Leu Gln Met
65 70 75 80

Asn Asn Leu Arg Gly Glu Asp Thr Ala Val Tyr Tyr Cys Ala Arg Asp
85 90 95

Pro Arg Arg Trp Thr Gln Leu Trp Ile Pro Pro Asp Tyr Trp Gly Gln
100 105 110

Gly Thr Leu Val Ser Val Ser Ser Ala Ser Thr Lys Gly Pro Ser Val
115 120 125

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 132 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Leu Leu Glu Ser Gly Gly Gly Gly Val Val Gln Pro Gly Gly Ser Leu
1 5 10 15

Arg Leu Ser Cys Glu Ala Ser Gly Phe Ser Leu Ile Asn Thr Ala Met
20 25 30

His Trp Val Arg Gln Ala Pro Xaa Lys Gly Pro Glu Trp Val Ser Val
35 40 45

Ser Ser Tyr Asp Gly Arg Glu Lys Tyr Tyr Thr Asp Ser Val Lys Gly
50 55 60

Arg Phe Ser Ile Ser Arg Asp Asp Ser Thr Asn Met Leu Tyr Leu Gln
65 70 75 80

Met Asn Ser Val Lys Ile Asp Asp Thr Ala Val Tyr Tyr Cys Ala Arg
85 90 95

Thr Asn Arg Ala Tyr Cys Ser Gly Val Arg Cys His Asp Gly Leu Asp
100 105 110

Val Trp Gly Gln Gly Thr Met Val Thr Val Ser Ser Ala Ser Thr Lys
115 120 125

Gly Pro Ser Val
130

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 133 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Lys Val
1 5 10 15

Ile Ile Ser Cys Ser Gly Ser Ser Ser His Thr Gly Gln Pro Lys Ala
20 25 30

Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu Glu Leu Gln Ala
35 40 45

Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe Tyr Pro Gly Ala
50 55 60

Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val Lys Ala Gly Val
65 70 75 80

Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn Lys Tyr Ala Xaa Ser
85 90 95

Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser His Lys Ser Tyr
100 105 110

Xaa Cys Gln Val Thr His Glu Gly Ser Thr Val Glu Lys Thr Val Xaa
115 120 125

Pro Thr Glu Cys Ser
130

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 111 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Lys Val
1 5 10 15

Thr Ile Ser Cys Ser Gly Ser Thr Ser Asn Ile Gly Asn Arg His Val
20 25 30

Ser Trp Tyr Gln Gln Leu Pro Gly Thr Xaa Pro Lys Leu Leu Ile Tyr
35 40 45

Glu Asn Asn Ile Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Ala Ser
50 55 60

Lys Ser Gly Thr Ser Ala Thr Leu Asp Ile Thr Gly Leu Gln Thr Gly
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gly Thr Trp Asp Ser Ser Leu Ser Thr
85 90 95

Gly His Trp Val Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Ser
100 105 110

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ala Glu Leu Gln Pro Pro Ser Val Ser Ala Ala Pro Gly Gln Ser Val
1 5 10 15

Thr Ile Ser Cys Ser Gly Ser Ser Ser Asn Ile Gly Asn Tyr Asn Val
20 25 30

Xaa Trp Tyr Gln Gln Thr Pro Gly Ser Ala Pro Lys Thr Leu Ile Tyr
35 40 45

Asp Thr His Lys Arg Pro Ser Gly Ile Ser Glu Arg Phe Ser Gly Ser
50 55 60

Lys Ser Gly Thr Ser Ala Thr Leu Gly Ile Thr Gly Leu Gln Thr Gly
65 70 75 80

Asp Glu Ala Asp Tyr Tyr Cys Gly Thr Trp Asp Thr Ser Leu Asn Ser
85 90 95

Ala Leu Phe Gly Gly Gly Thr Lys Leu Thr Xaa Leu Gly
100 105

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 109 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Glu Leu Gln Asp Pro Val Val Ser Val Ala Leu Gly Gln Thr Val
1 5 10 15

Arg Met Thr Cys Gln Gly Asp Ser Leu Arg Tyr His Tyr Ala Asn Trp
20 25 30

Tyr Gln Gln Lys Pro Gly Gln Ala Pro Ile Leu Val Ile Lys Ser Lys
35 40 45

Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser
50 55 60

Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu
65 70 75 80

Ala Asp Tyr Tyr Cys Asp Ser Arg Asp Thr Ser Gly Asn His Pro Arg
85 90 95

Val Leu Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly
100 105

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ala Glu Leu Gln Asp Pro Val Val Ser Val Ala Leu Gly Gln Thr Val
1 5 10 15

```
Arg Ile Thr Cys Gln Gly Asp Ser Leu Arg Xaa Tyr His Ala Asn Trp
                20                  25                  30

Tyr Gln Gln Lys Pro Gly Lys Ala Pro Ile Phe Val Ile Tyr Gly Lys
        35                  40                  45

Asn Asn Arg Pro Ser Gly Ile Pro Asp Arg Phe Ser Gly Ser Ser Ser
    50                  55                  60

Gly Asn Thr Ala Ser Leu Thr Ile Thr Gly Ala Gln Ala Glu Asp Glu
65                  70                  75                  80

Ala Asp Tyr Tyr Cys Asn Ser Arg Asp Ser Ser Ser Asn His Val Val
                85                  90                  95

Phe Gly Gly Gly Thr Lys Leu Thr Val Leu Gly
            100                 105
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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 106 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Gly Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser
1               5                   10                  15

Glu Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp
            20                  25                  30

Phe Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro
        35                  40                  45

Val Lys Ala Gly Val Glu Thr Thr Thr Pro Ser Lys Gln Ser Asn Asn
    50                  55                  60

Lys Tyr Ala Xaa Ser Ser Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys
65                  70                  75                  80

Ser His Lys Ser Tyr Xaa Cys Gln Val Thr His Glu Gly Ser Thr Val
                85                  90                  95

Glu Lys Thr Val Xaa Pro Thr Glu Cys Ser
            100                 105
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 58 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

Gln Pro Lys Xaa Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Xaa Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Phe Pro Gly Xaa Xaa Xaa Val Xaa Trp Lys Xaa Asp Ser Xaa Pro Xaa
35 40 45

Lys Gly Gly Val Glu Thr Thr Xaa Pro Pro
50 55

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 71 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Gln Pro Lys Xaa Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Xaa Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Xaa Thr Val Xaa Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Ala Gly Val Glu Asn Thr Thr Pro Ser Ile Xaa Met Gln Gln Gln
50 55 60

Val Ser Gly Pro Gly Gly Ile
65 70

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 88 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Val Thr Val Ala Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Gly Gly Val Glu Thr Thr Thr Pro Ser Asn Gln Ser Asn Asn Lys
50 55 60

Phe Ala Ala Ser Arg Tyr Leu Ser Leu Thr Pro Glu Gln Trp Lys Ser
65 70 75 80

His Arg Ser Tyr Ser Cys Gln Val
85

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 98 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Gln Pro Lys Ala Ala Pro Ser Val Thr Leu Phe Pro Pro Ser Ser Glu
1 5 10 15

Glu Leu Gln Ala Asn Lys Ala Thr Leu Val Cys Leu Ile Ser Asp Phe
20 25 30

Tyr Pro Gly Ala Val Xaa Val Ala Trp Lys Ala Asp Ser Ser Pro Val
35 40 45

Lys Val Gly Val Xaa Xaa Thr Thr Pro Ser Xaa His Xaa Ile Asn Met
50 55 60

Phe Ala Gly Ser Xaa Tyr Leu Ser Leu Thr Pro Glu Gln Trp Xaa Ser
65 70 75 80

His Arg Lys Leu Gln Leu Pro Gly Gln Arg Arg Met Xaa Gly Ala Pro
85 90 95

Xaa Arg

What is claimed is:

1. In a method for conducting gene therapy wherein a recombinant gene is introduced into cells of a mammal, the improvement comprising using a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with a disease.

2. The improvement of claim 1 wherein the recombinant gene is free of a secretion sequence for said antibody.

3. The improvement of claim 1 wherein the recombinant gene encodes a single chain antibody.

4. The improvement of claim 1 wherein the recombinant gene encodes a single binding domain.

5. The improvement of claim 1 wherein the recombinant gene encodes a multiple binding domain.

6. The improvement of claim 1 wherein the recombinant gene includes an intracellular localization signal.

7. The improvement of claim 1 wherein the recombinant gene encodes an antibody that is selectively specific for an intracellular viral antigen.

8. The improvement of claim 1 wherein the recombinant gene encodes an antibody that is selectively specific for an intracellular antigen associated with the human immunodeficiency virus.

9. The improvement of claim 1 wherein the recombinant gene is part of an infectious agent that is replication-defective.

10. A method for preventing or halting the progress of a disease comprising administering to the subject a recombinant gene in an infectious vector, the gene encoding an antibody that is selectively specific for an intracellular antigen associated with the intracellular pathogen.

11. A method as claimed in claim 10 further characterized by administering a gene that is free of a secretion sequence for said antibody.

12. A method as claimed in claim 10 wherein the antibody is selectively specific for a viral antigen.

13. A method as claimed in claim 10 wherein the recombinant gene includes an intracellular localization signal.

14. A method as claimed in claim 10 wherein the infectious vector is replication-defective.

15. A method as claimed in claim 10 wherein the antibody is a single chain antibody.

16. A method as claimed in claim 10 wherein the antibody is a single chain antibody that is selectively specific for a human immunodeficiency virus antigen.

17. A method as claimed in claim 10 wherein the antibody comprises a single binding domain.

18. A method as claimed in claim 10 wherein the antibody comprises a multiple binding domain.

19. A method for preventing or halting the progression of a disease in a subject caused by an intracellular pathogen comprising introducing into cells *ex*

vivo a recombinant gene encoding an antibody that is selectively specific for an antigen associated with the pathogen to form immunized cells, and introducing the immunized cells into the subject.

20. A method as claimed in claim 19 wherein the cells are isolated from the subject prior to forming the immunized cells.

21. A method for inhibiting replication of an intracellular pathogen in a cell, comprising causing to be introduced into the cell a recombinant gene encoding an antibody that is selectively specific for an intracellular antigen associated with the pathogen.

22. A method as claimed in claim 21 wherein the recombinant gene is part of an infectious agent and wherein the recombinant gene is introduced into the cell by contacting the cell with the infectious agent.

23. A method as claimed in claim 21 wherein the recombinant gene is free of a secretion sequence for said antibody.

24. A method as claimed in claim 21 wherein the recombinant gene encodes a single chain antibody.

25. A method as claimed in claim 21 wherein the recombinant gene encodes a single binding domain.

26. A method as claimed in claim 21 wherein the recombinant gene encodes a multiple binding domain.

27. A method as claimed in claim 22 wherein the infectious agent is replication defective.

28. A method as claimed in claim 21 wherein the recombinant gene includes an intracellular localization sequence.

29. A method as claimed in claim 21 wherein the pathogen is a human immunodeficiency virus.

30. A viral vector comprising a single chain antibody gene encoding an antibody directed against an essential function of another virus, wherein expression of said antibody gene causes inhibition of said essential function thereby affecting replication of said other virus.

31. The viral vector of claim 30 being a retrovirus.

32. The viral vector of claim 30 being an adeno associated virus.

33. The viral vector of claim 30 being a Semliki Forest virus.

34. The viral vector of claim 30, wherein said other virus is human immunodeficiency virus type 1.

35. The viral vector of claim 34, wherein said single chain antibody gene is directed against human immunodeficiency virus type 1 rev.

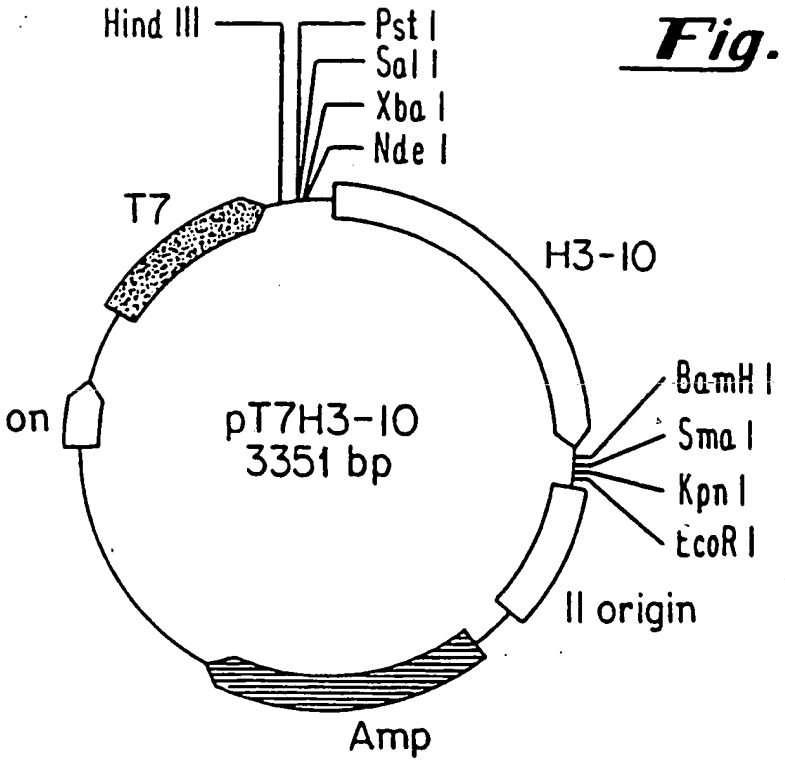


Fig. 1

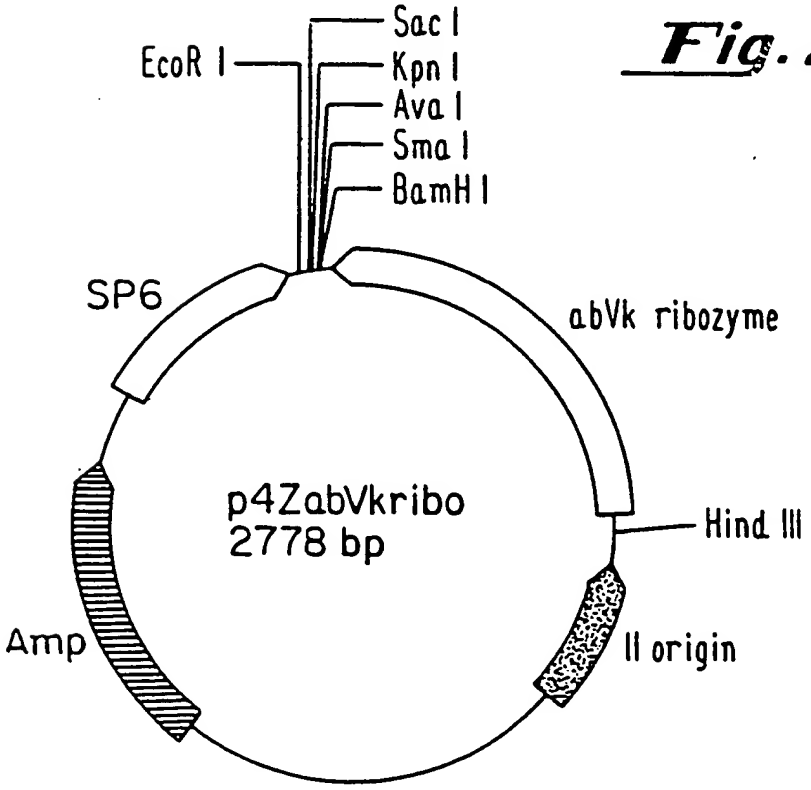
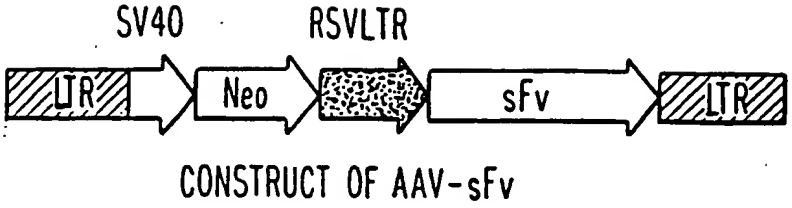
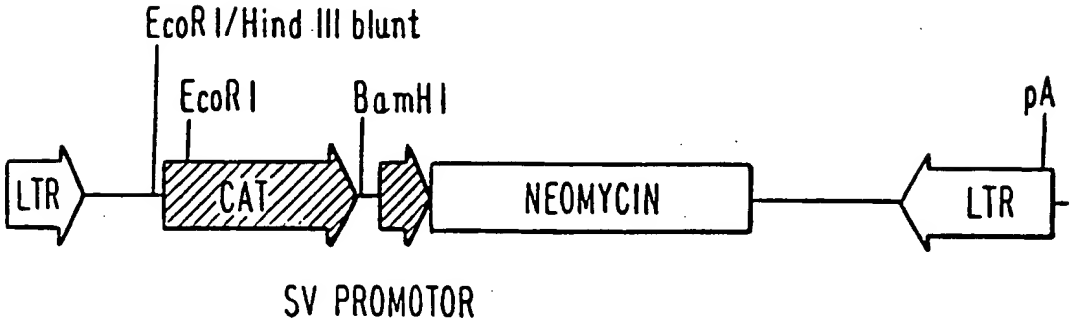


Fig. 2



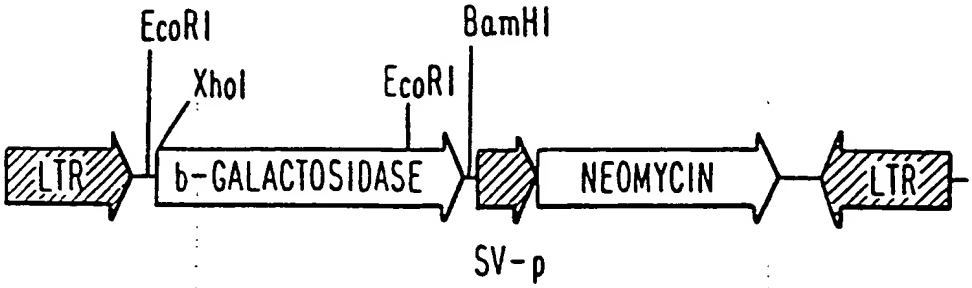
CONSTRUCT OF AAV-sFv

Fig. 3



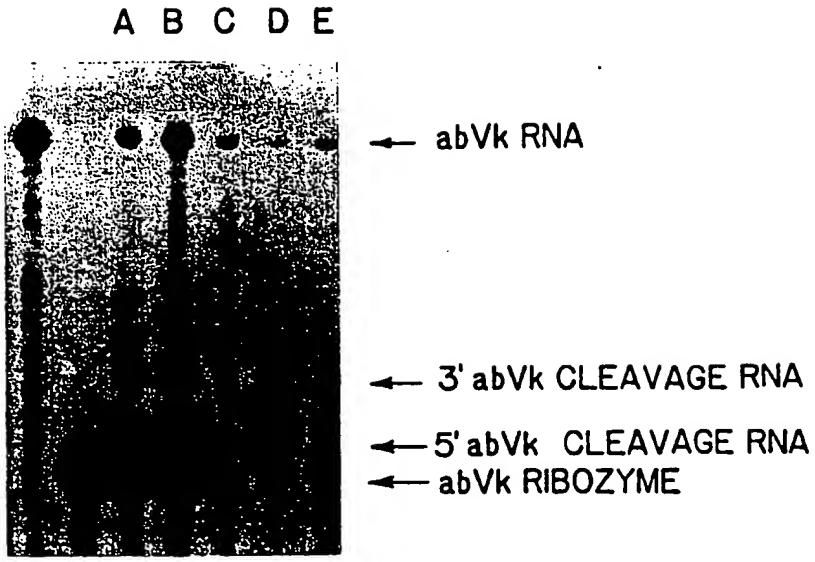
SV PROMOTOR

Fig. 5



SV-p

Fig. 6



A: REACTION IN 5 x RT BUFFER
B: REACTION IN 4 x RT BUFFER
C: REACTION IN 3 x RT BUFFER
D: REACTION IN 2 x RT BUFFER
E: REACTION IN 1 x RT BUFFER

FIG. 4

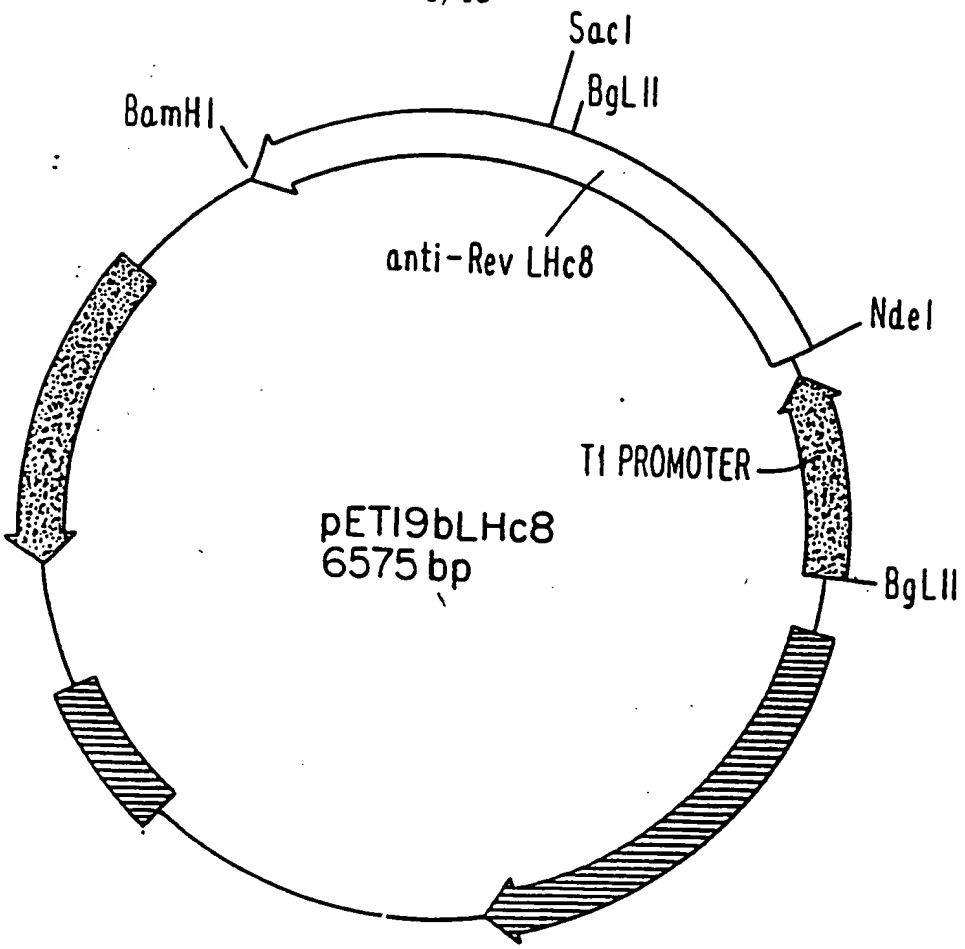


Fig. 7

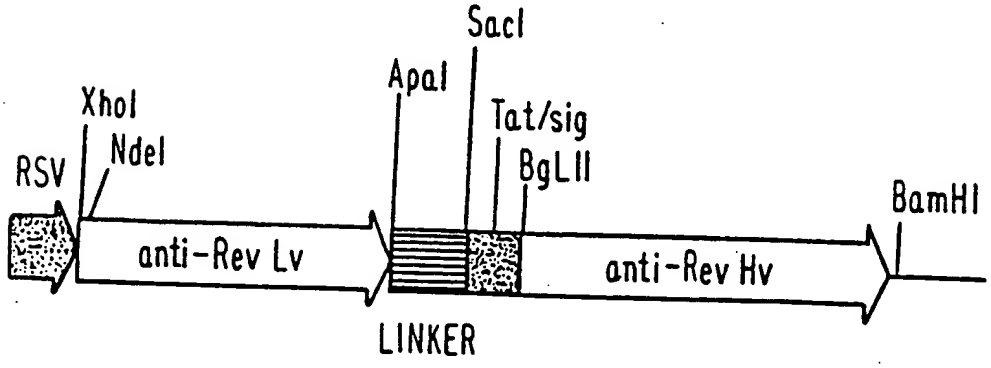


Fig. 8

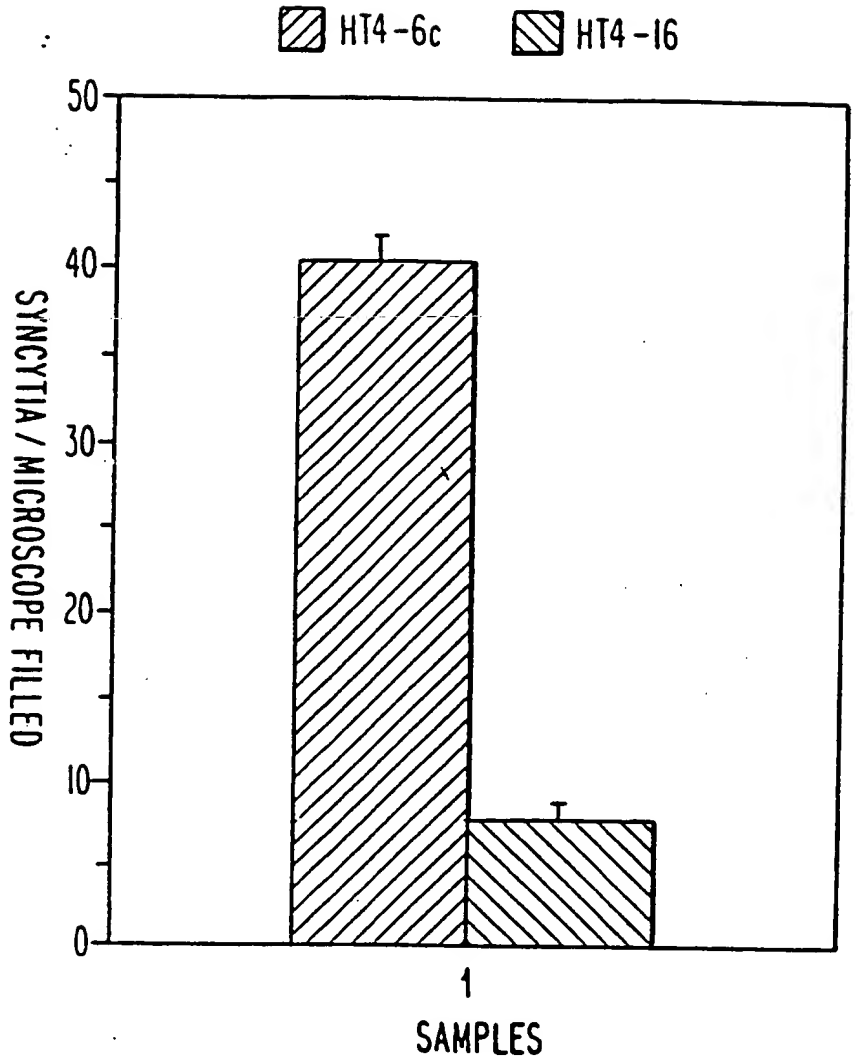


Fig. 9

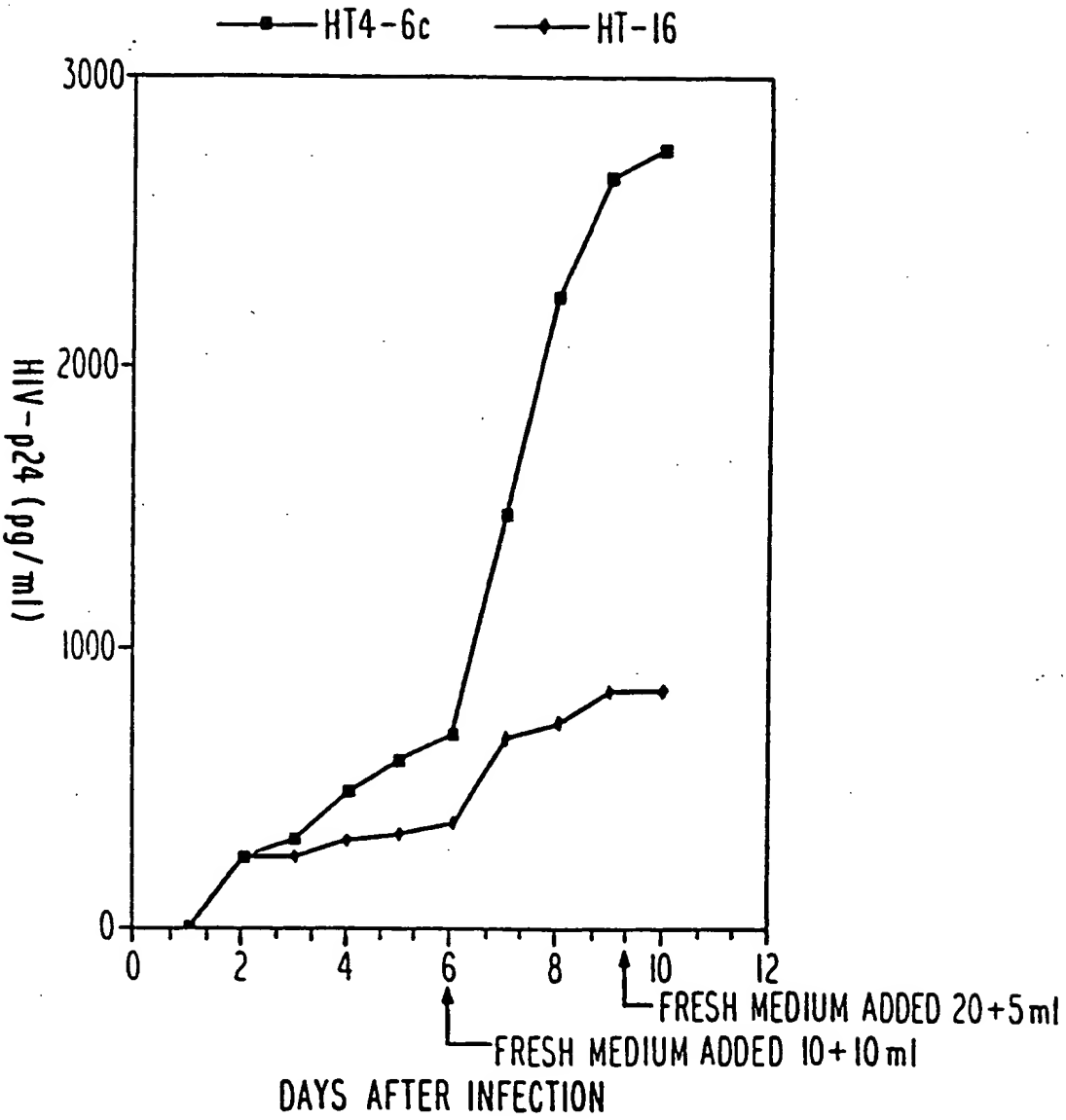


Fig. 10

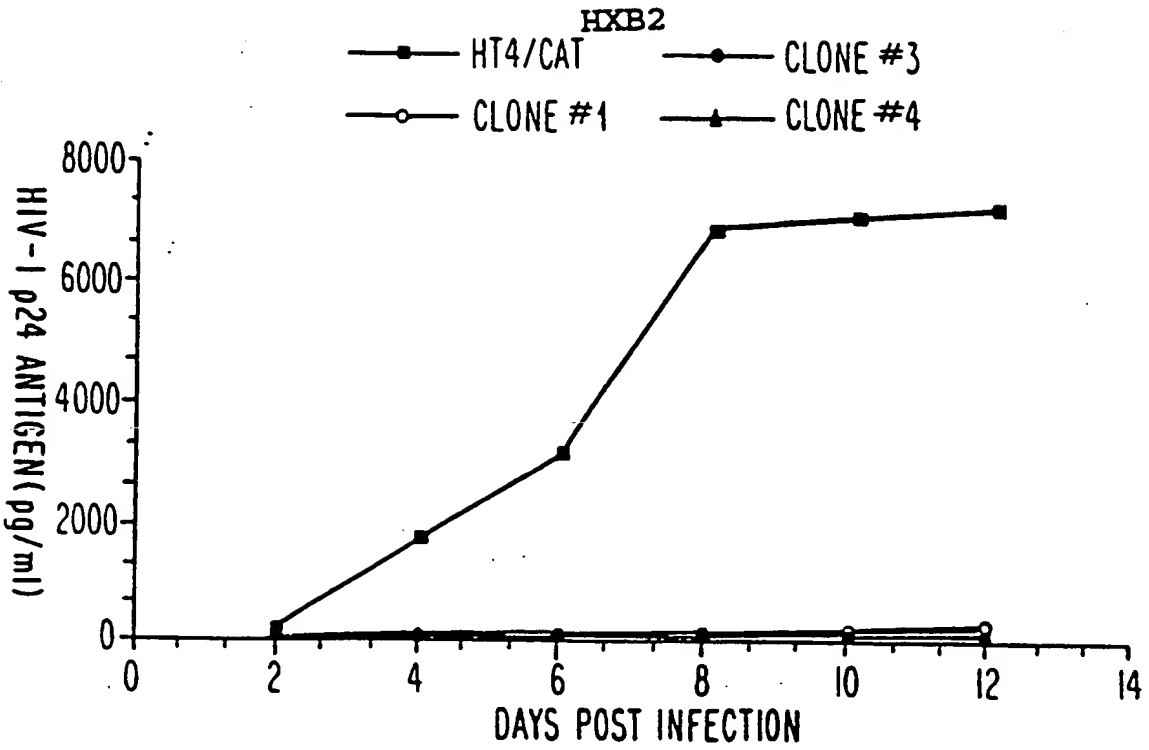


Fig. IIa

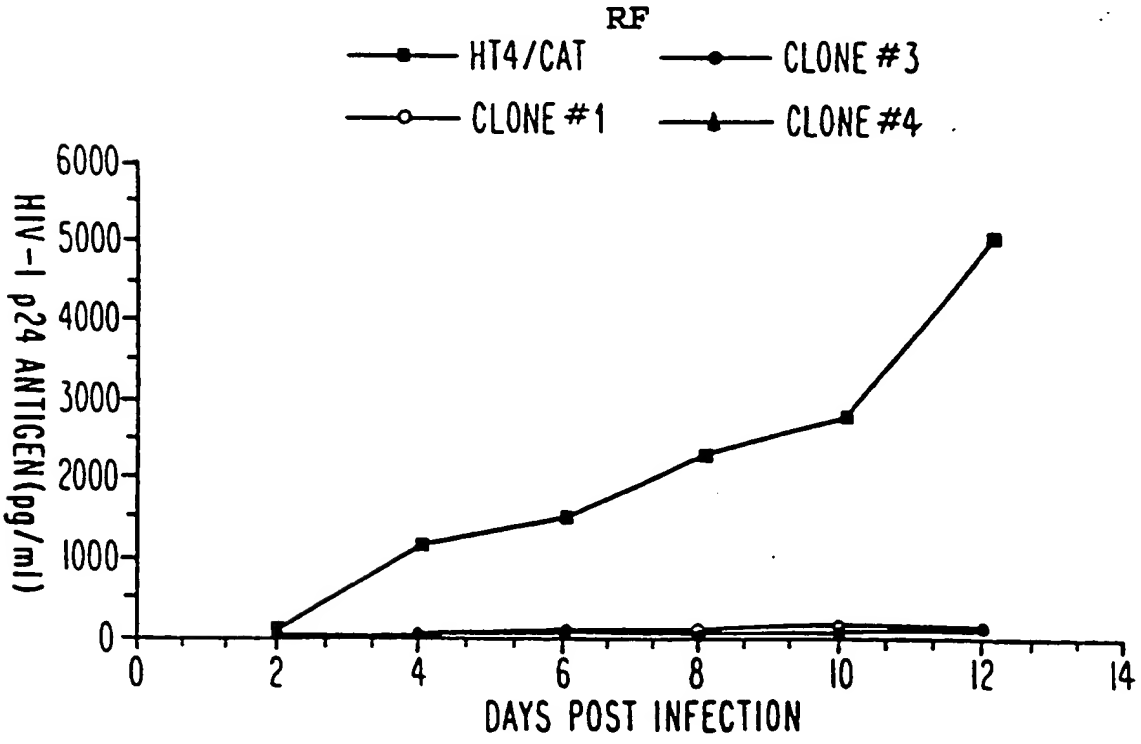


Fig. IIb

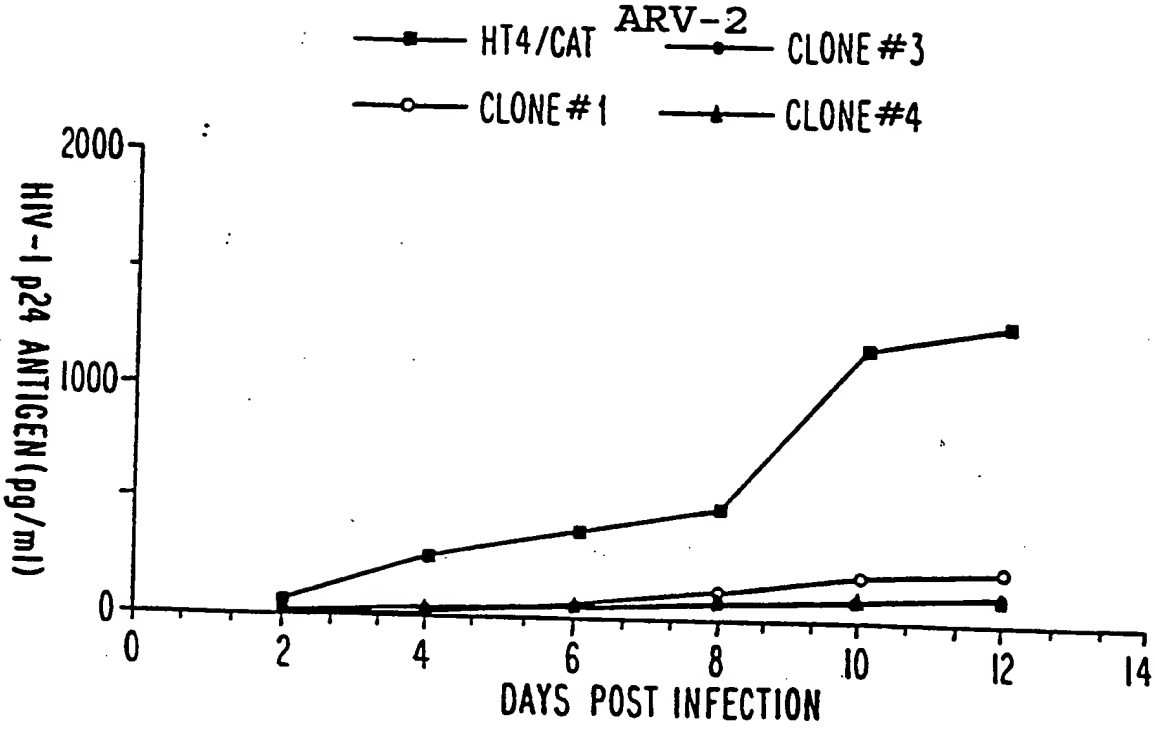


Fig. IIc

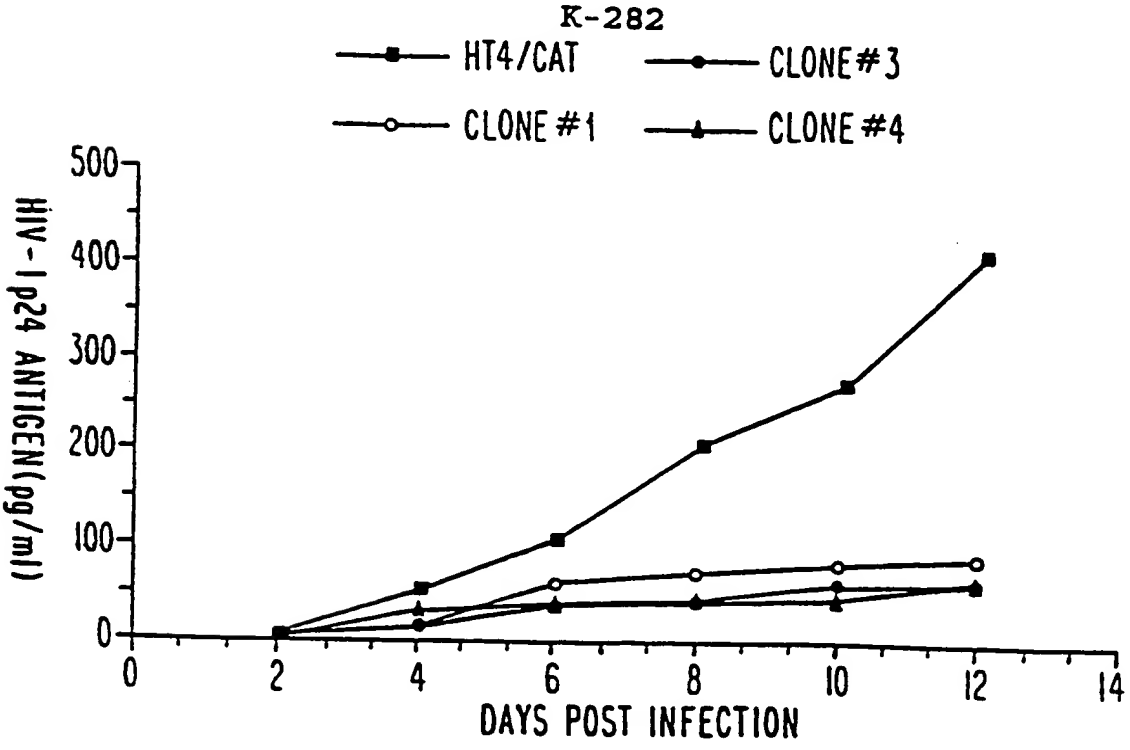


Fig. IId

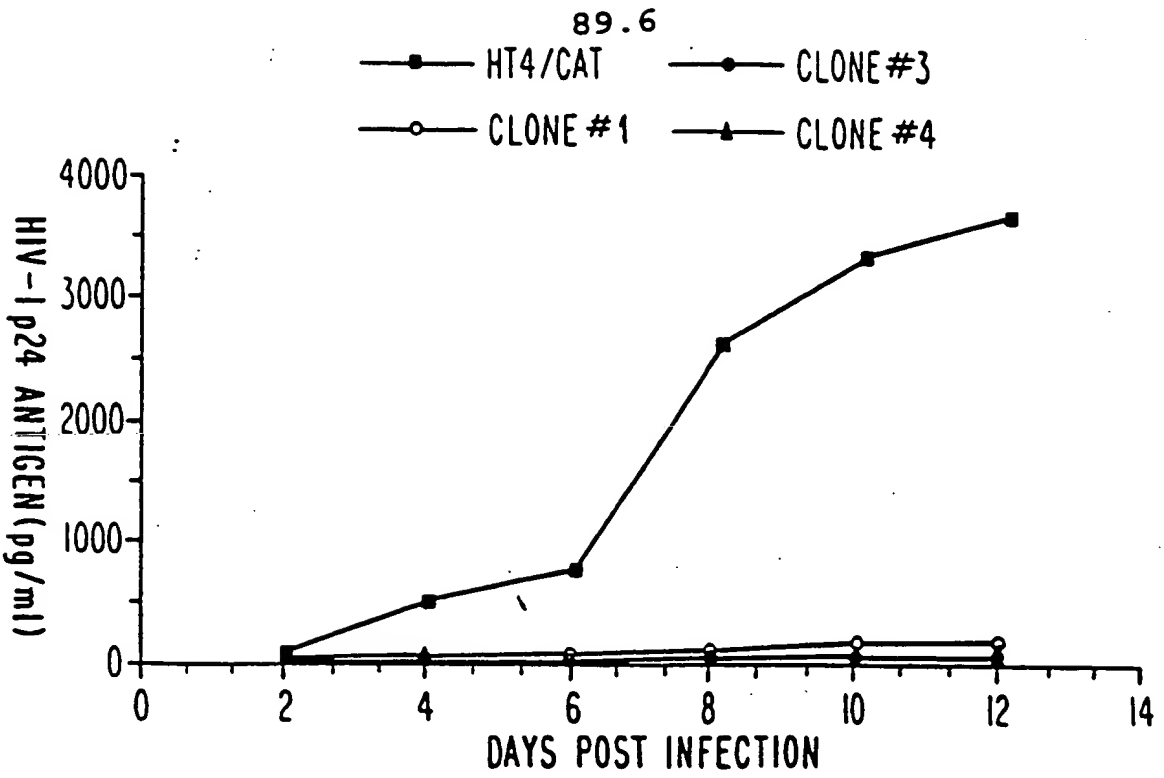


Fig. IIe

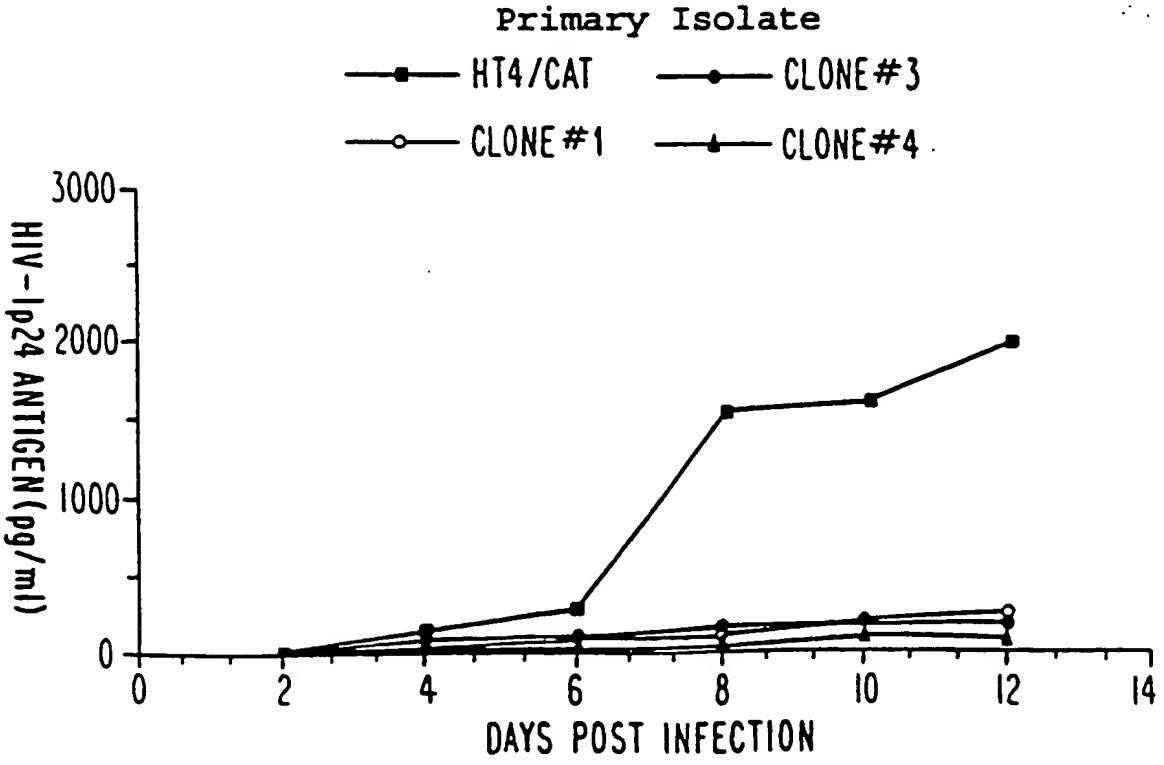


Fig. IIf

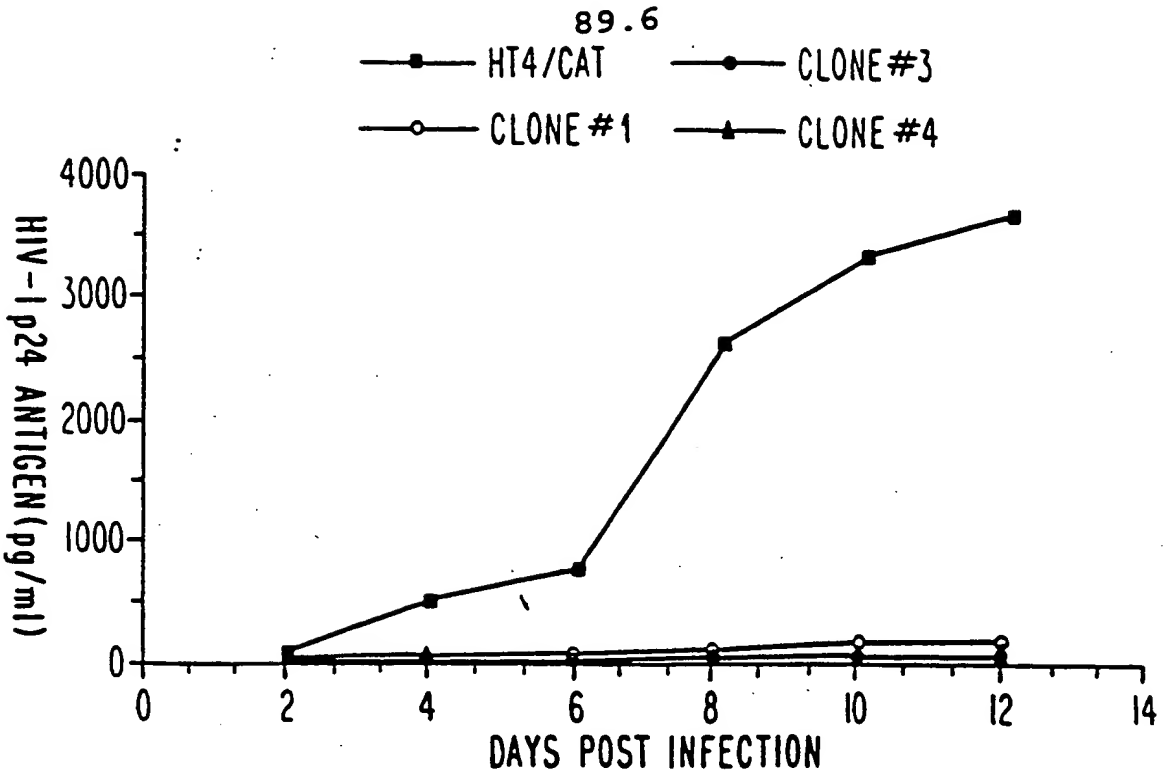


Fig. IIe

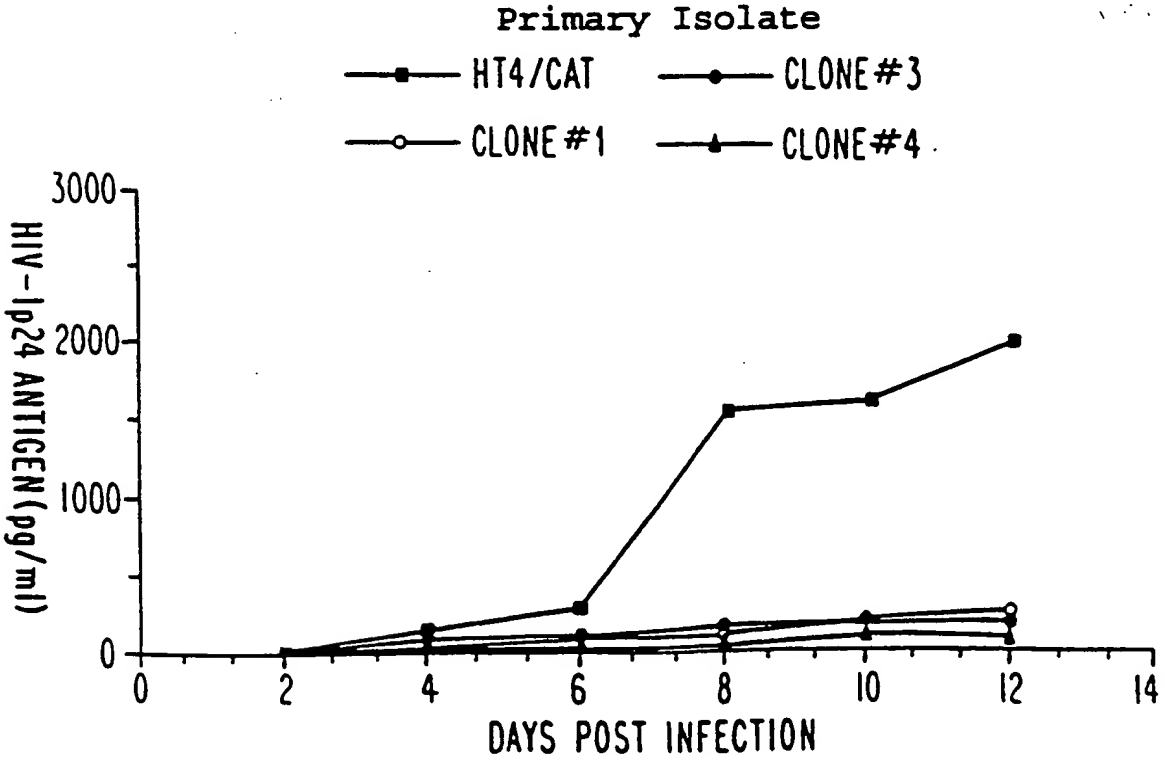


Fig. IIf

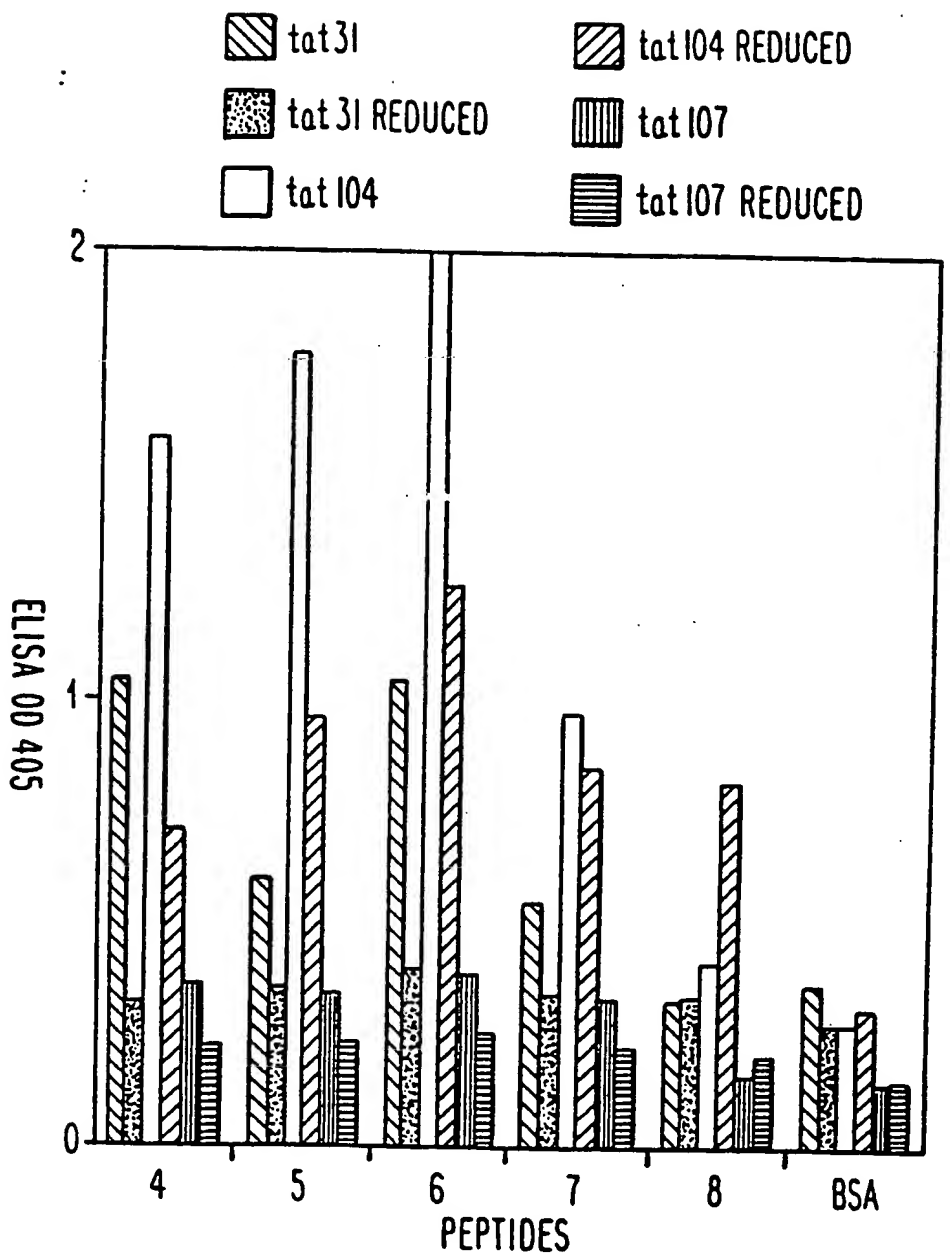


Fig. 13

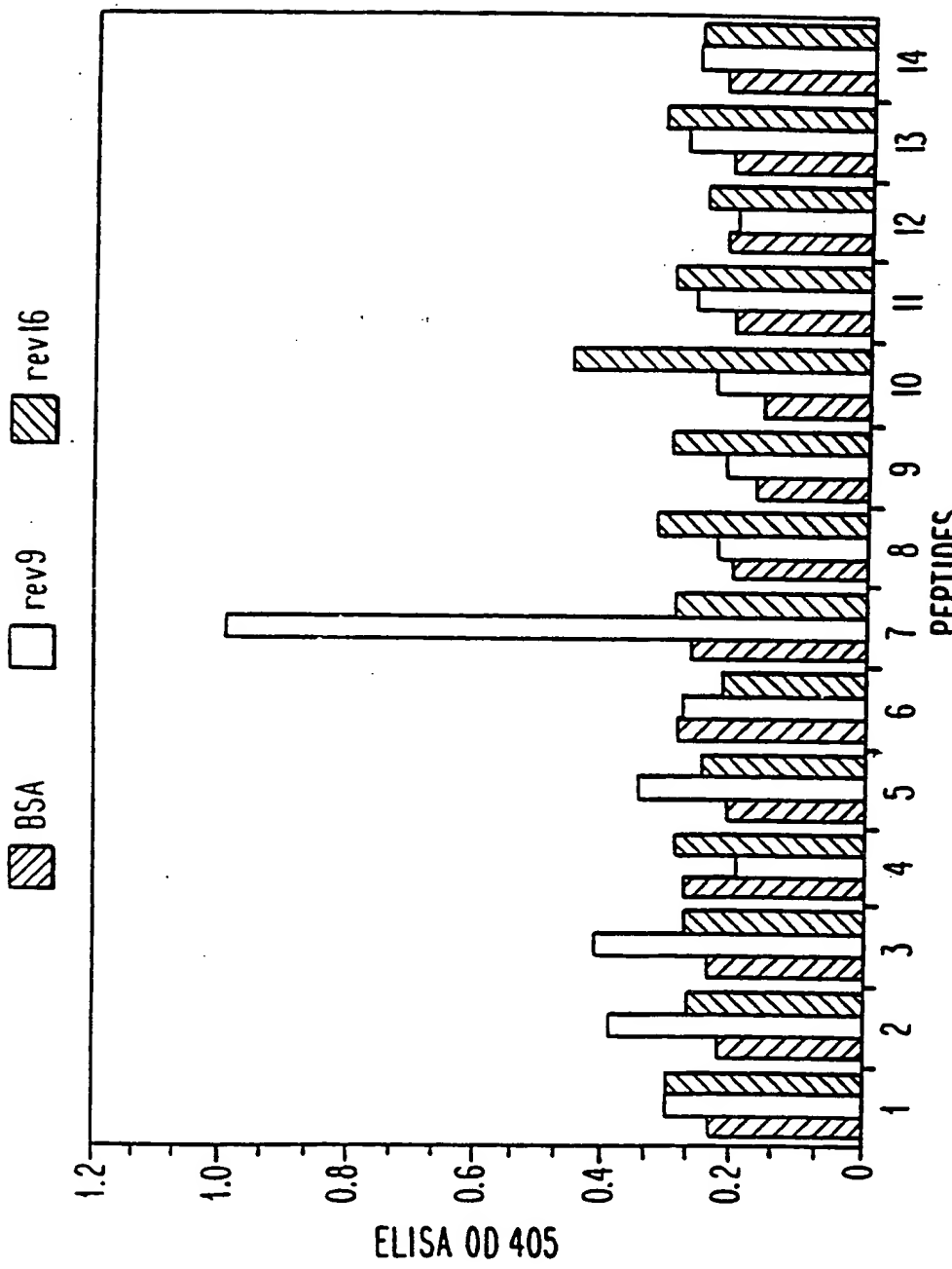


Fig. 14

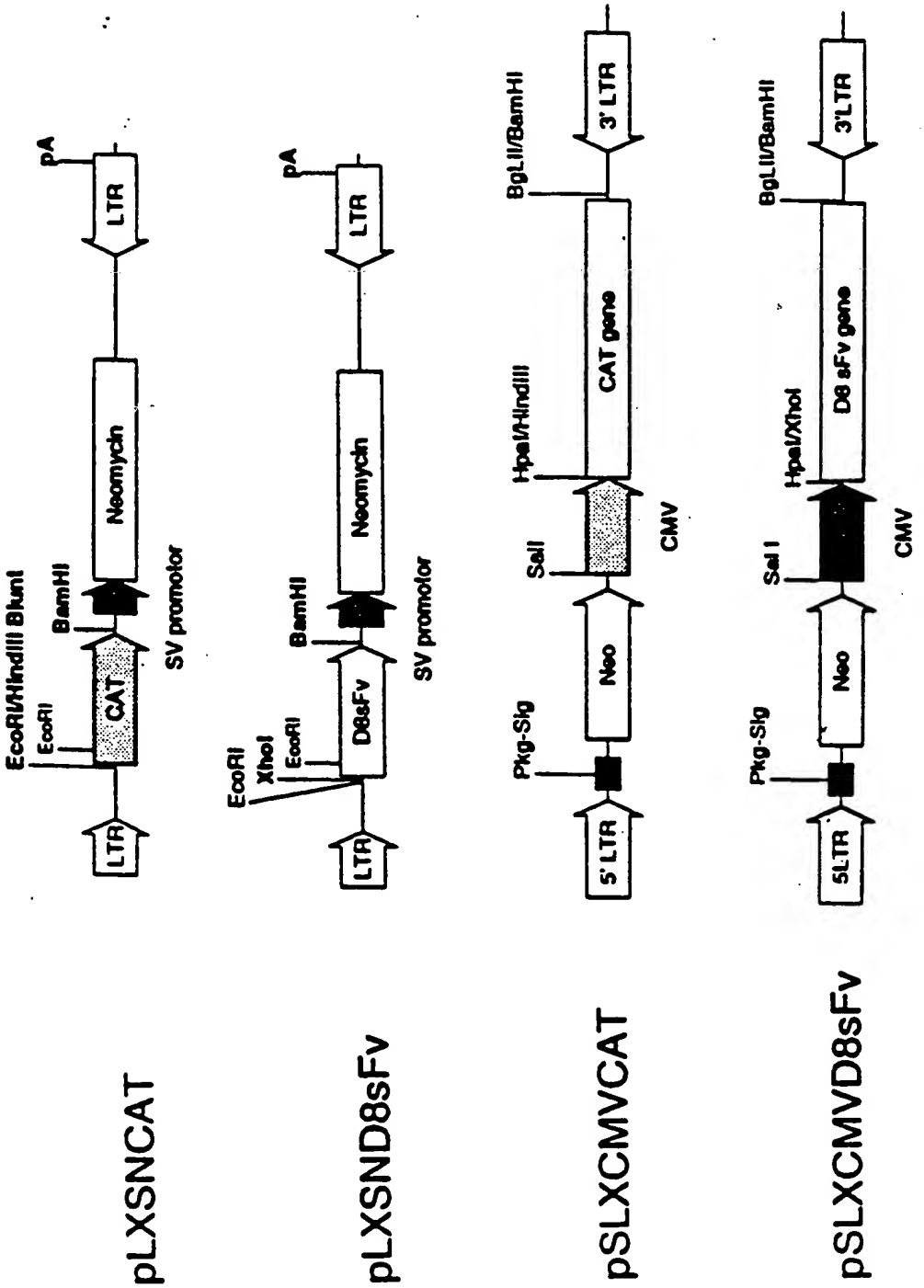


FIG. 15

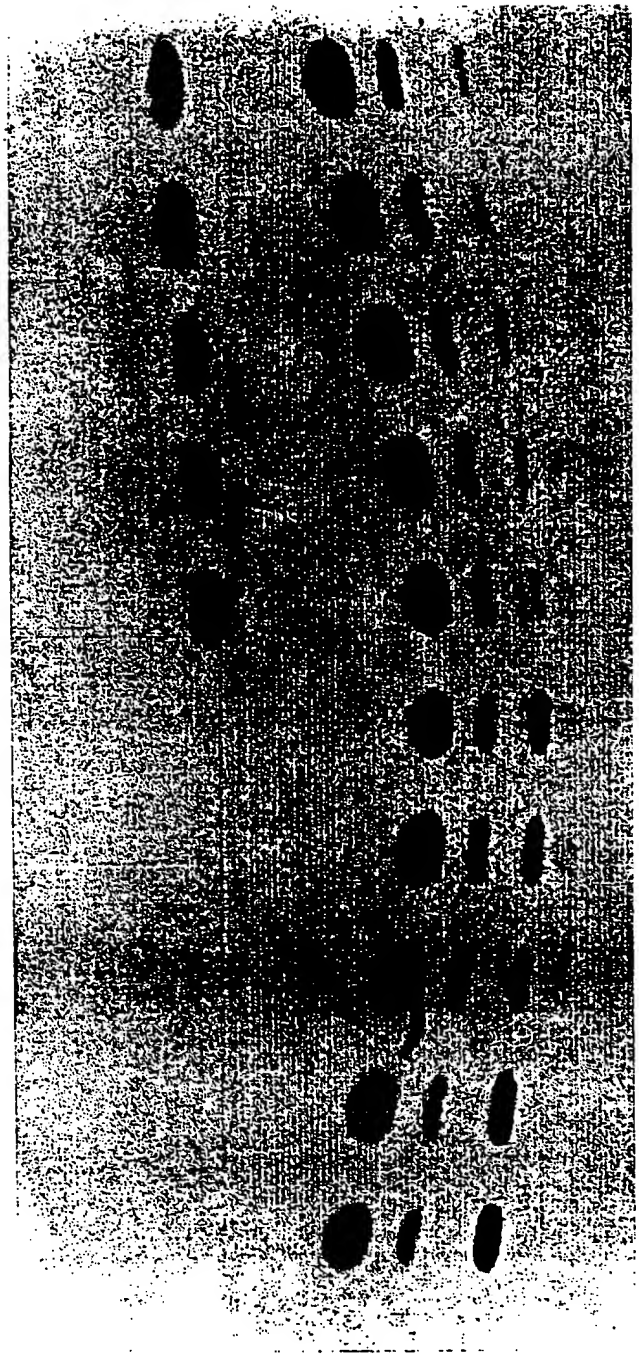


FIG.16.

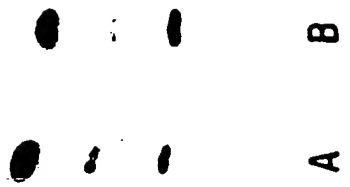


FIG.17B

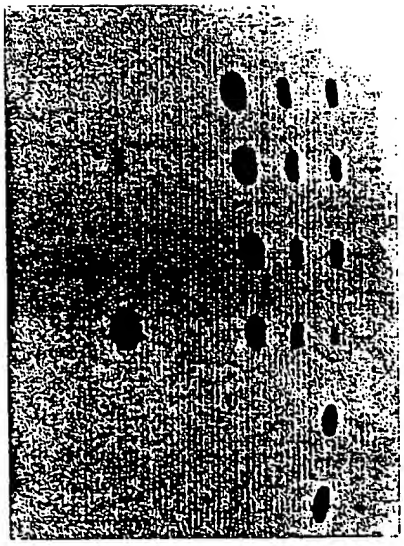


FIG.17A

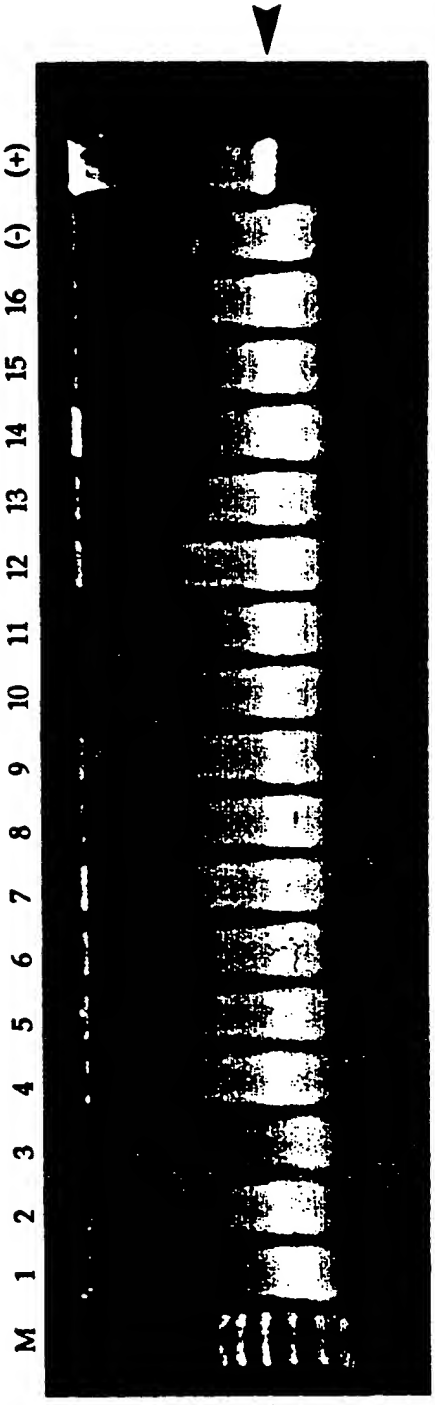


FIG.18A

FIG.18B

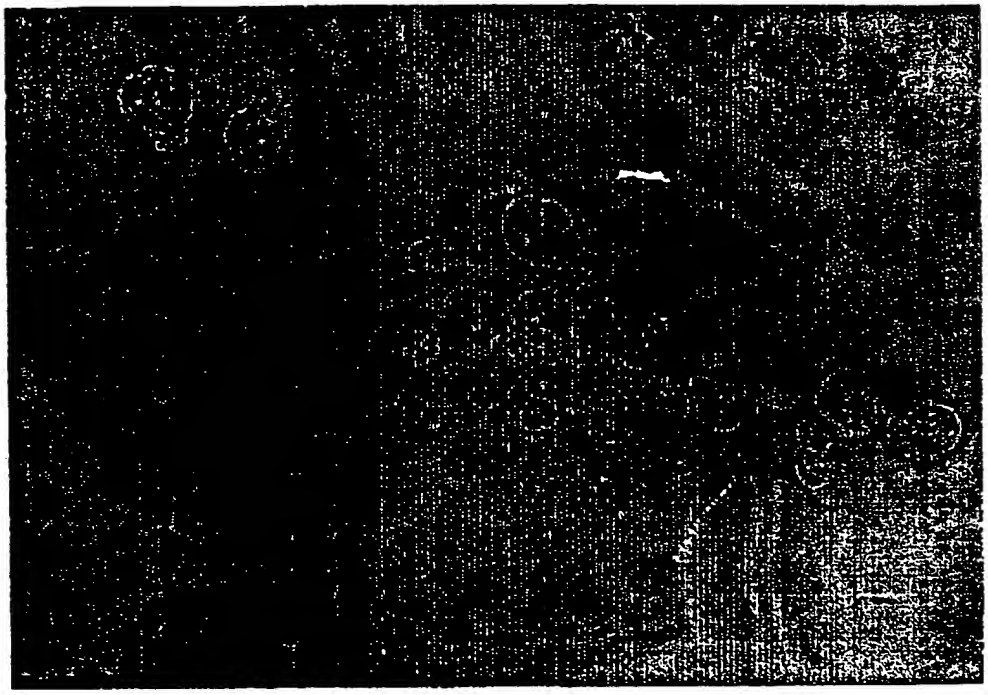


FIG.18C

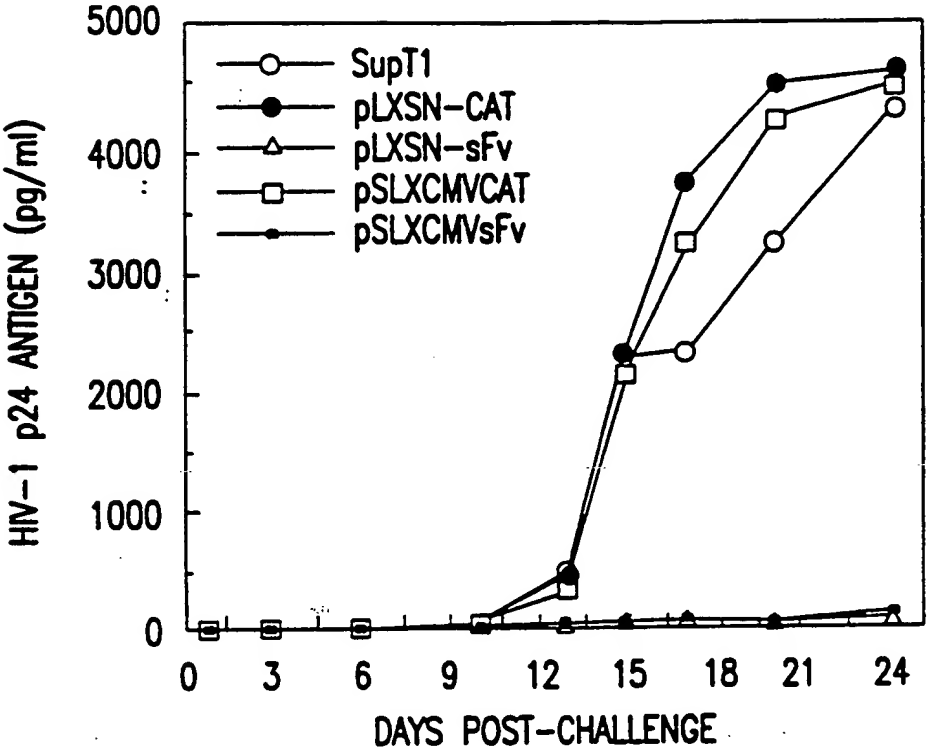


FIG.19A

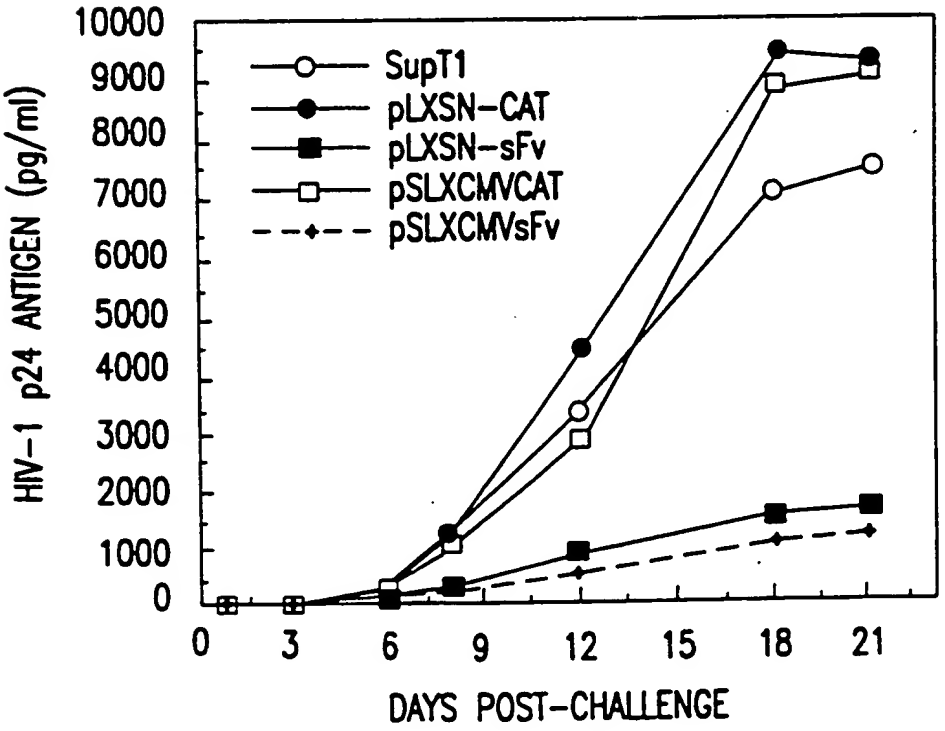


FIG.19B

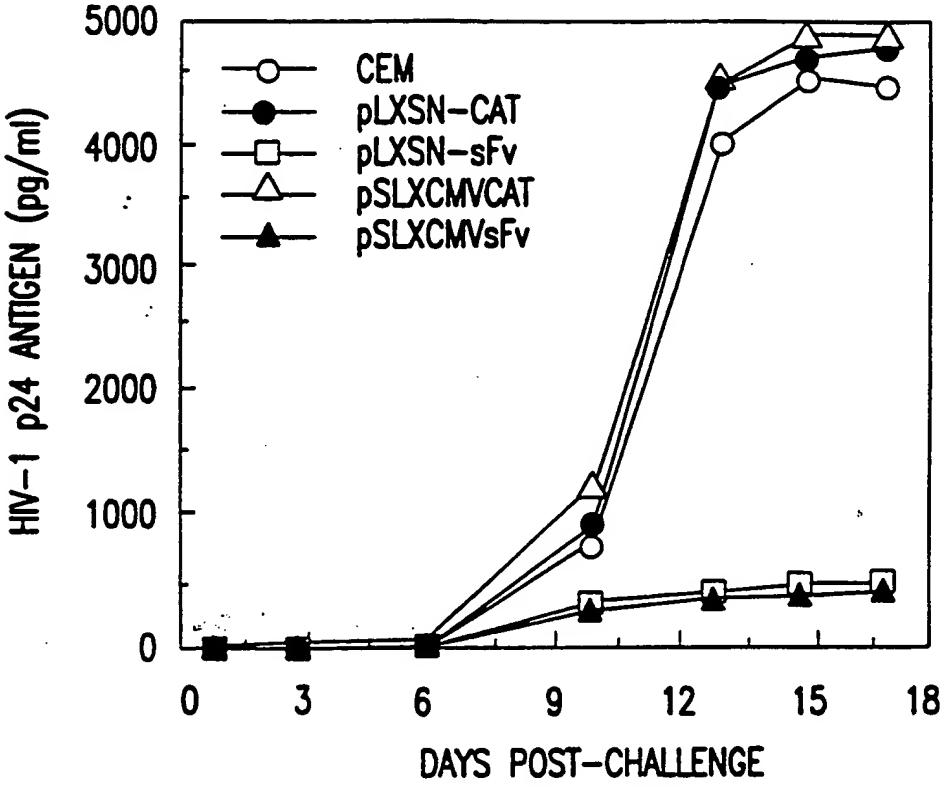


FIG.19C

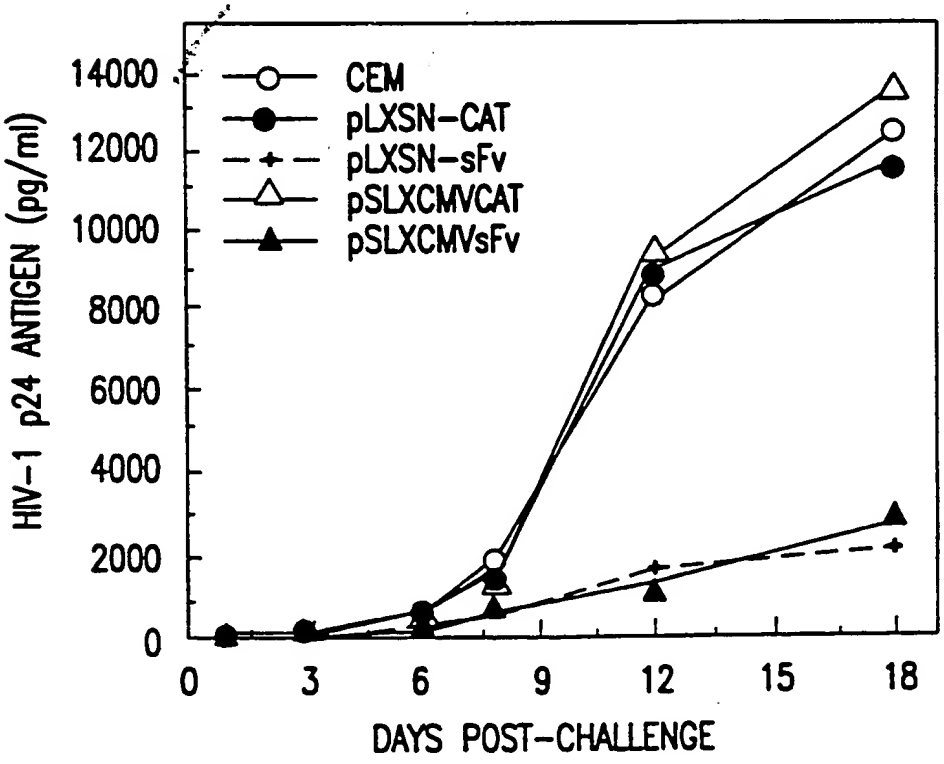


FIG.19D

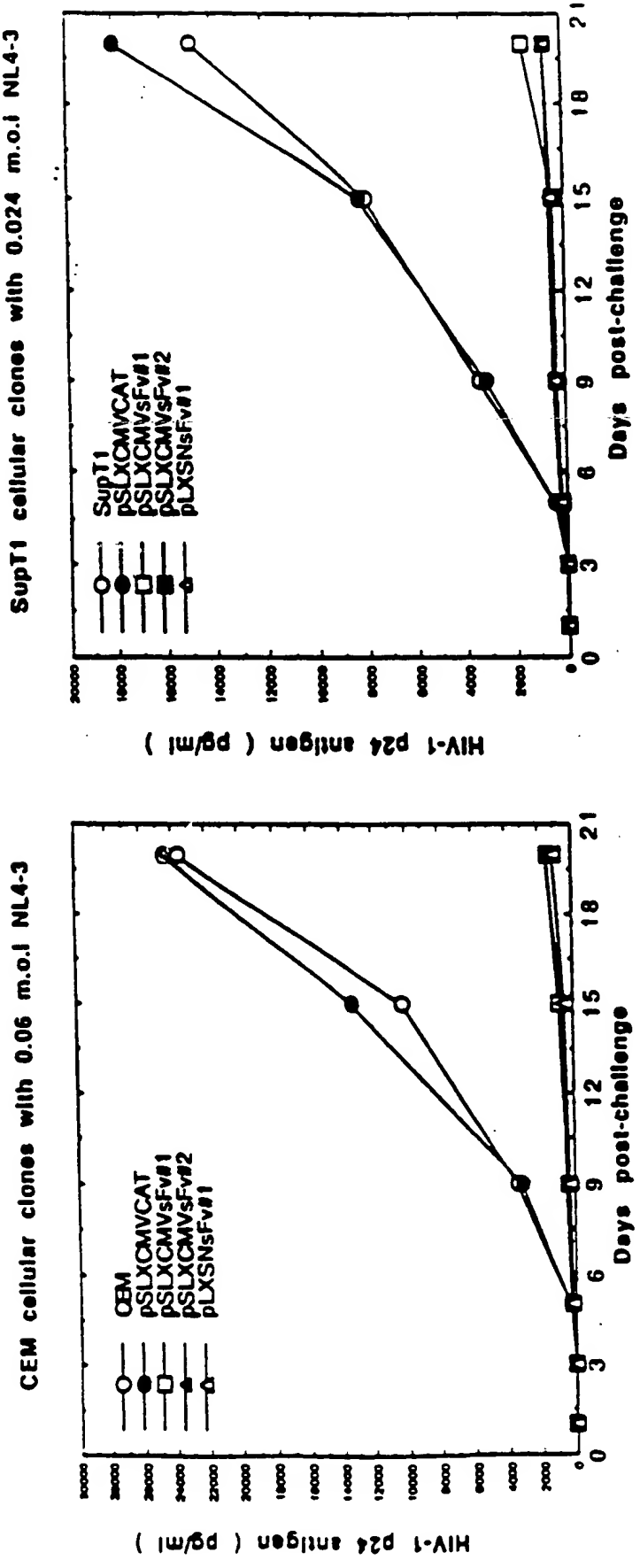


FIG. 20

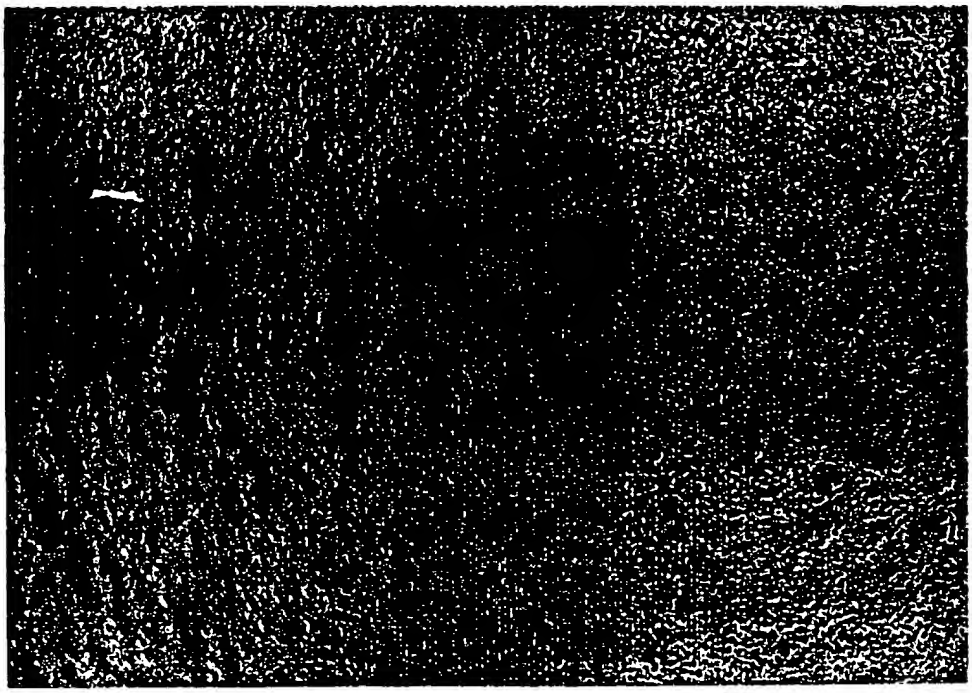


FIG.21A

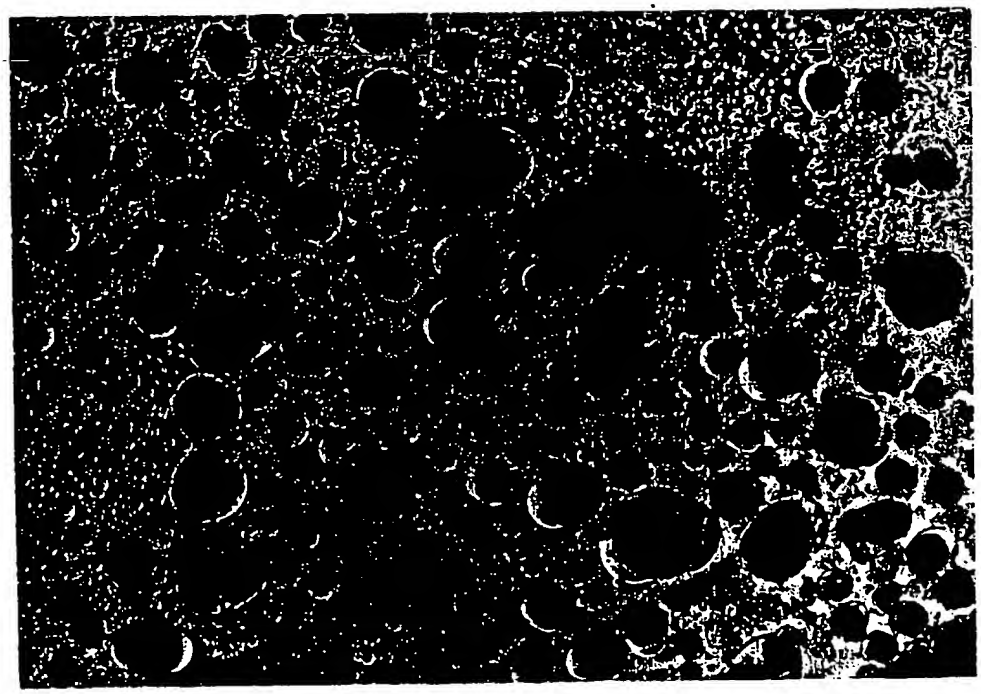
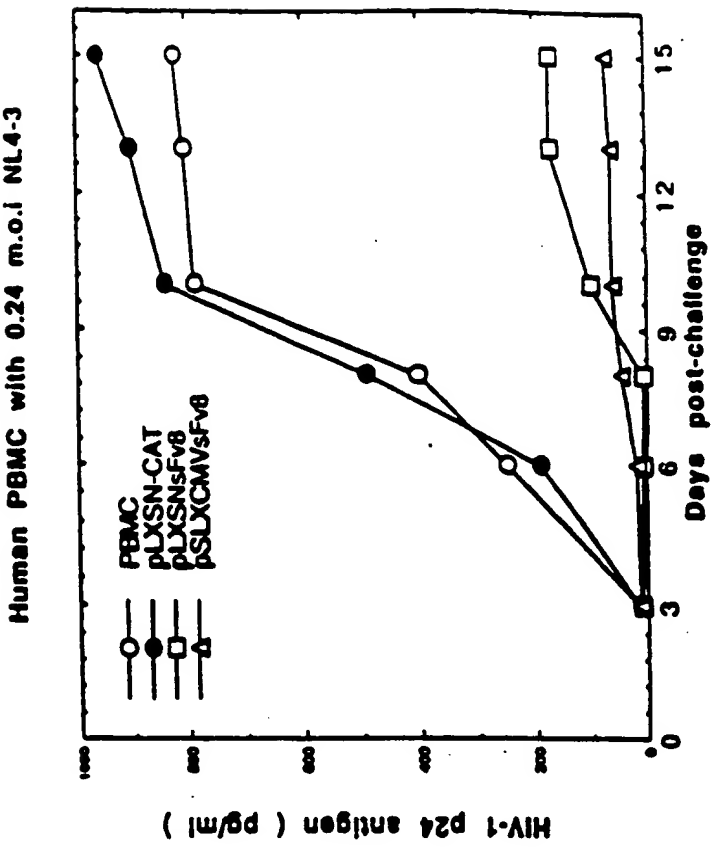


FIG.21B



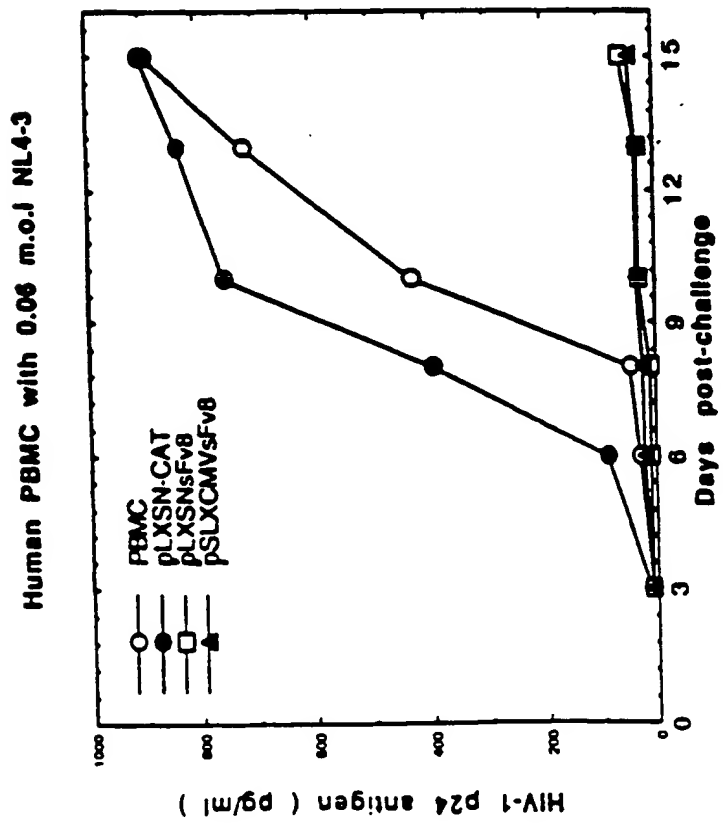


FIG. 22

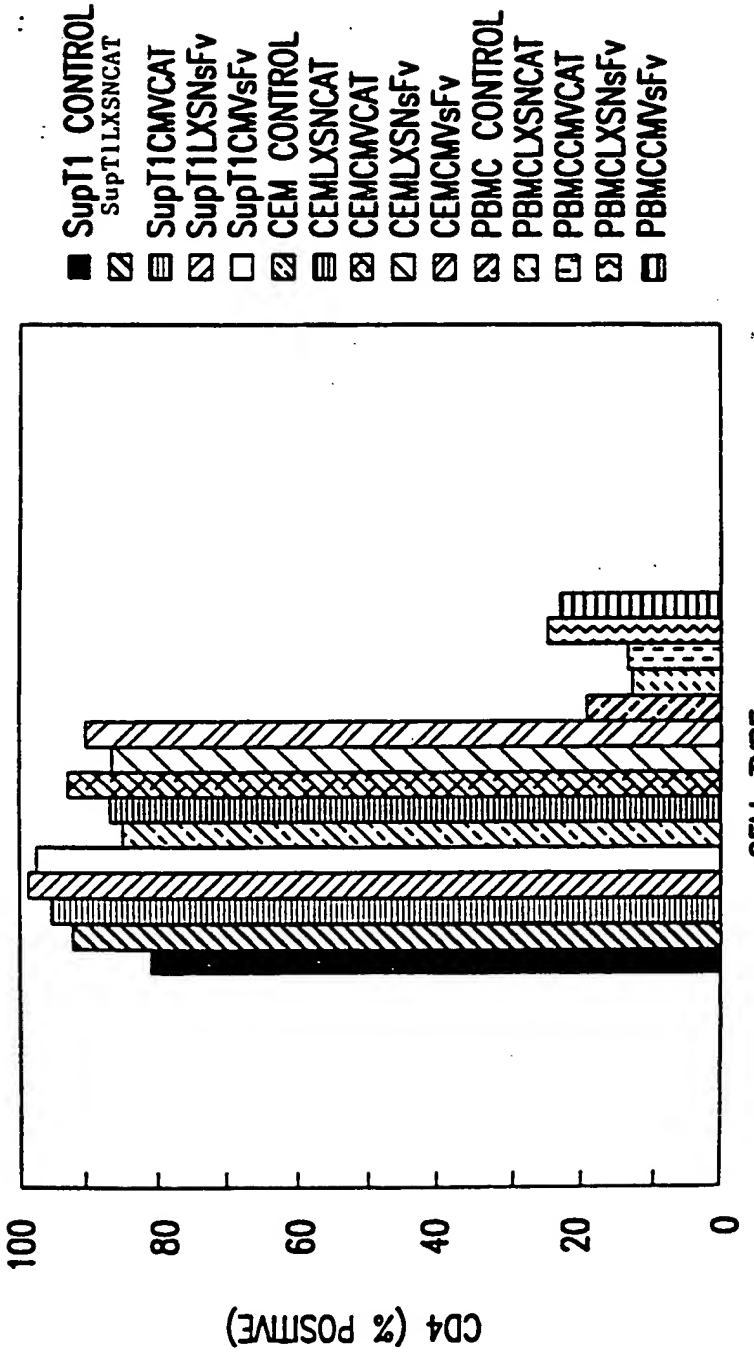


FIG.23A

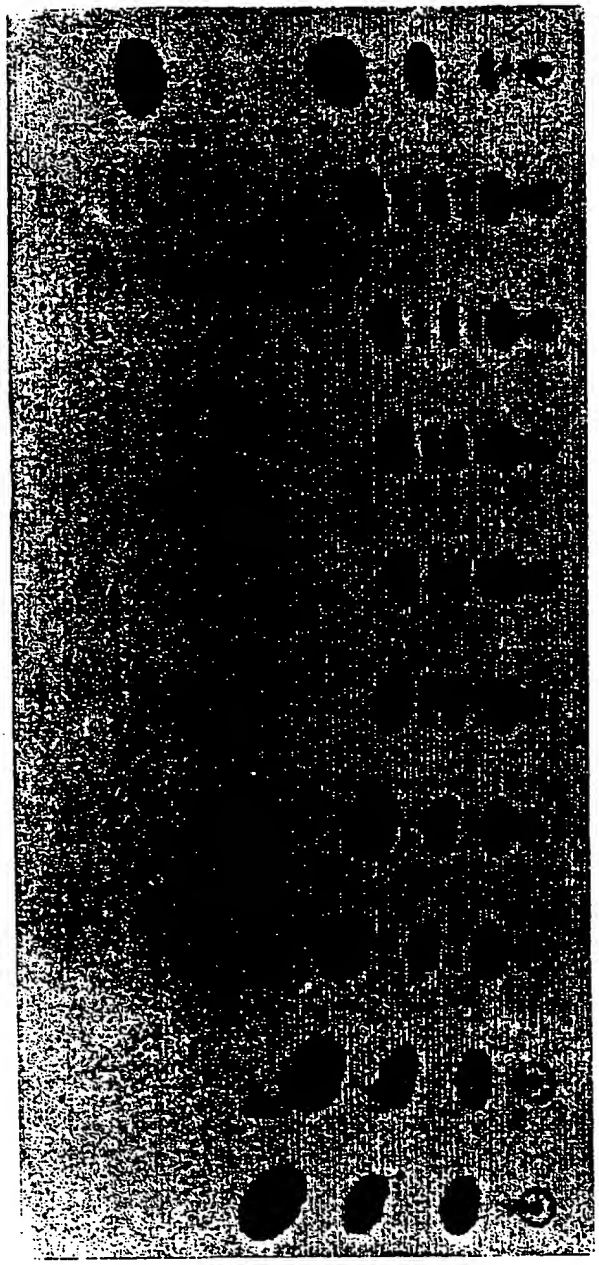


FIG.23B

Figure 24A

anti-Rev sFv Transduced Human PBMC Challenged With a Primary Isolate of HIV-1 (SI)

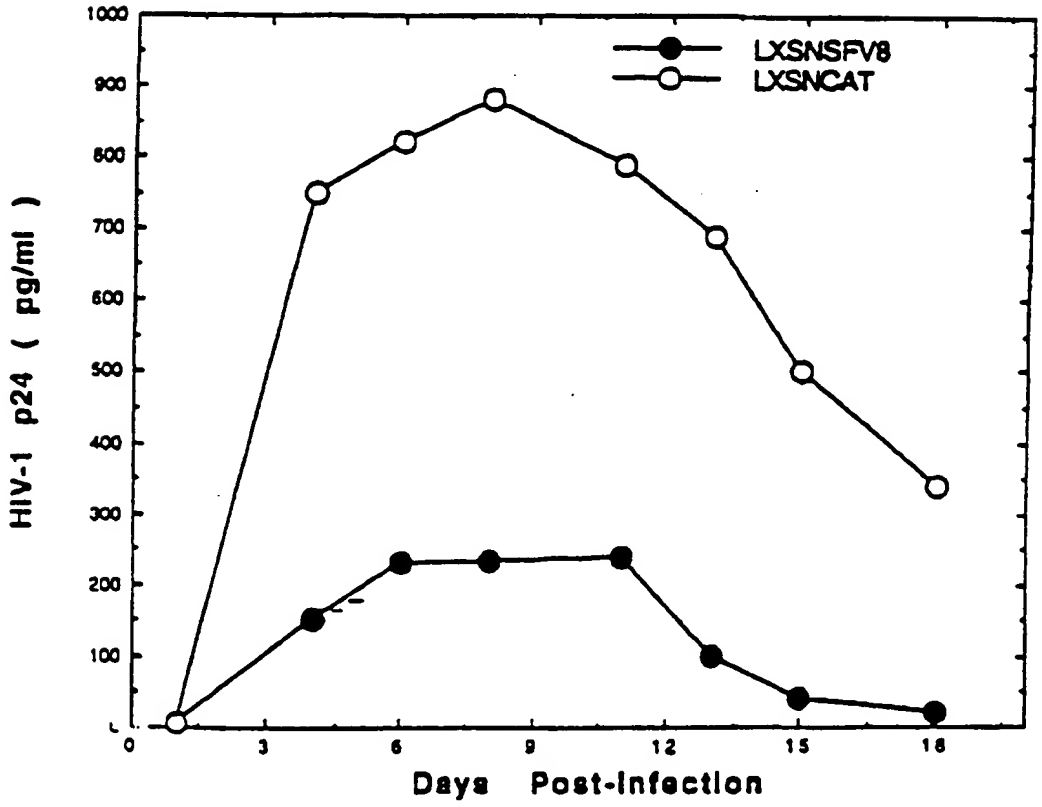


Figure 24B

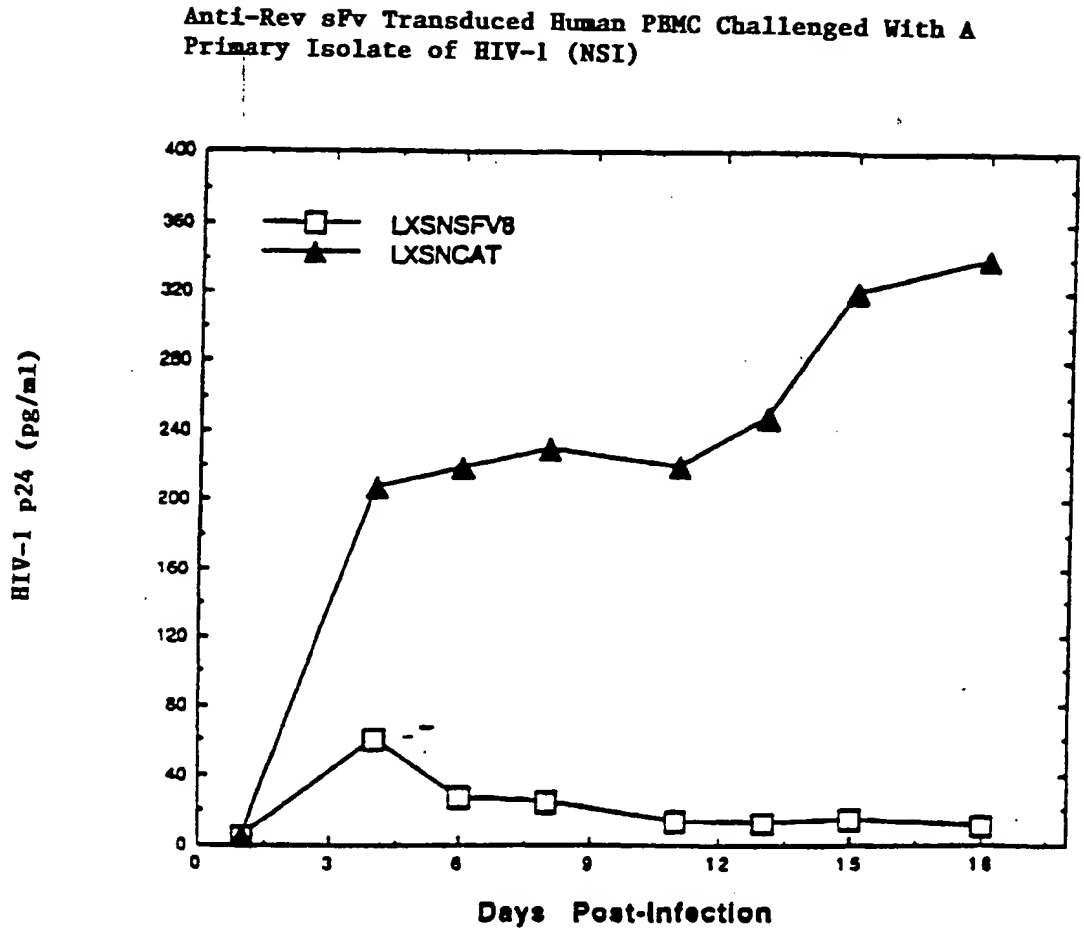


Figure 25

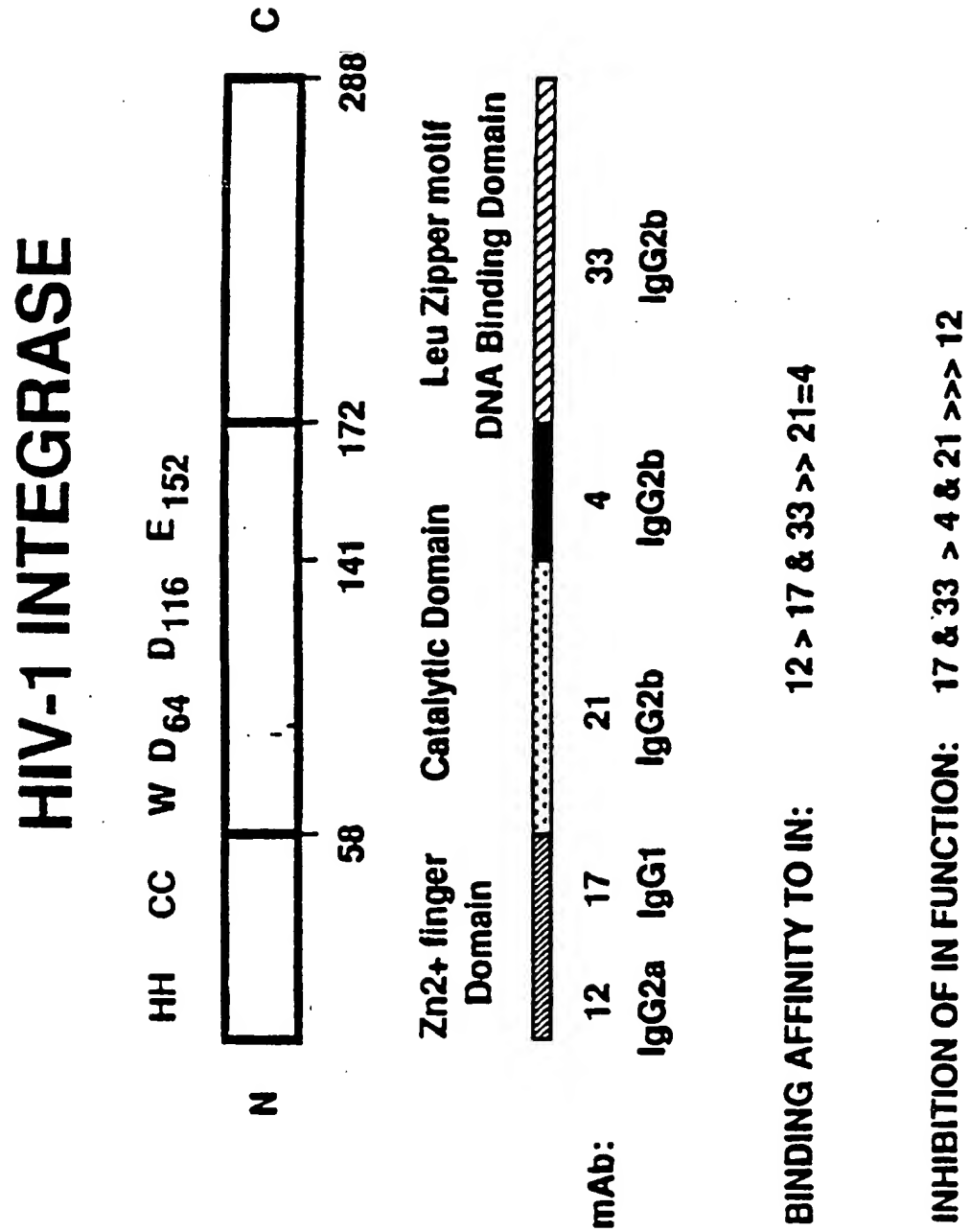
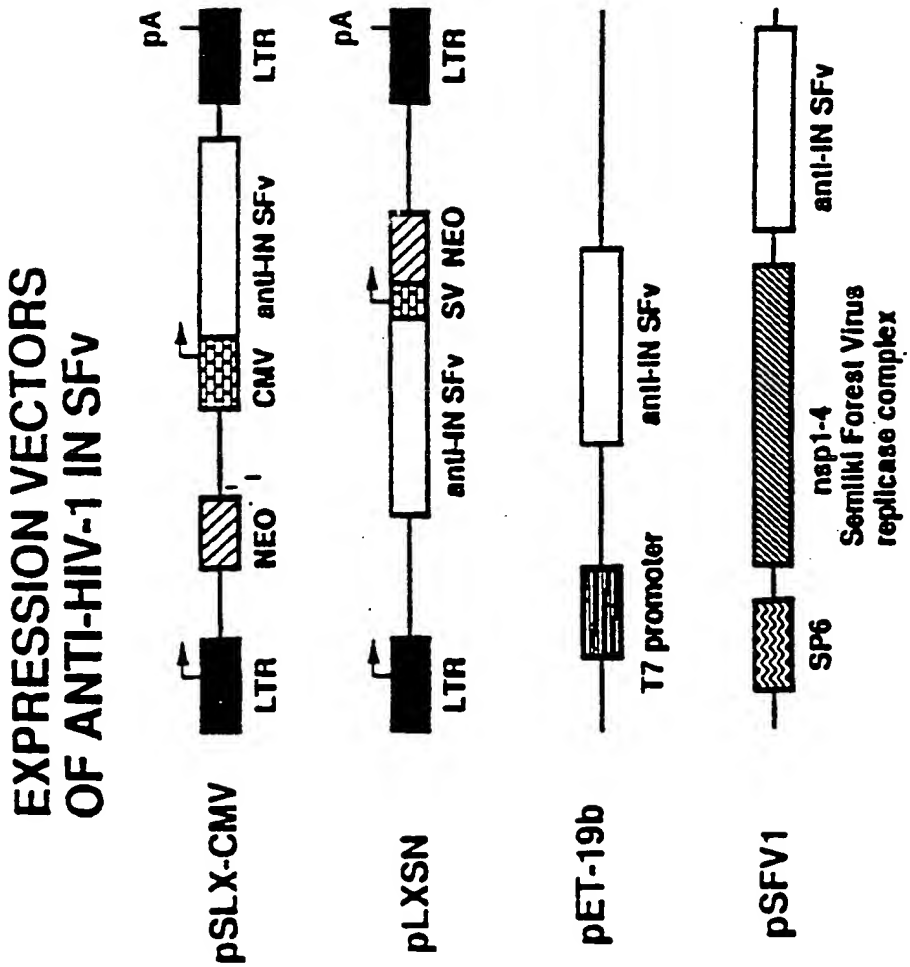


Figure 26



```
  1 ATGGAGCGGG GTCTTTCTCT CCTCCTGTCA GTAATTGCAG GTGTCCAATC CCAGGTTCAA
 61 CTGCAGCAGT CTGGGGCTGA GCTGGTGAGG CCTGGGGCTT CAGTGAGCCT GTCCTGCAAG
121 GCTTCGGGCT ACACATTTAC TGACTATGAA ATGCACTGGG TGAAGCAGAC ACCTGTGCAT
181 GGCCTGGAAT GGATTGGAGC TATTGATCCT GAAACTAGTG GTACTGCCTA CAATCAGAAC
241 TTCAAGGGCA AGGCCACACT GACTGCAGAC AGATCCTCCA GCACAGCCTA CATGGAGCTC
301 CGCAGCCTGA CATCTGAGGA CTCTGCCGAC TATTACTGTA CAAGAGGGTT TGCTTACTGG
361 GGCCAAGGGA CTCTGGTCAC TGTGTCTGCA GCCGAAACGA CACC
```

FIG. 27A

```
        10        20        30        40        50        60
    *   *   *   *   *   *   *   *   *   *   *   *
MERGLSLLLS VIAGVQSQVQ LQQSGAELVR PGASVTLSCK ASGYTFTDYE MHWVKQTPVH
        70        80        90       100       110       120
    *   *   *   *   *   *   *   *   *   *   *   *
GLEWIGAIDP ETSGTAYNQN FKGKATLTAD KSSSTAYMEL RSLTSEDSAD YYCTRGFAYW
       130
    *   *
GQGTLVTVSA AETT
```

FIG. 27B

```
      60         70         80         90         100        110
   *   *   *   *   *   *   *   *   *   *   *   *
GATGTTGTG ATGACCCAGA CTCCACTCAC TTTGTCGGTT ACCATTGGAC AACCAGCCTC

      120        130        140        150        160        170
   *   *   *   *   *   *   *   *   *   *   *   *
CATCTCTTGC AAGTCAAGTC TGAGCCTCTT AGATAGTGAT GGAAAGACAT ATTTGAATTG

      180        190        200        210        220        230
   *   *   *   *   *   *   *   *   *   *   *   *
GTTGTTACAG CGGCCAGGCC AGTCTCCAAA GCGCCTAATC TATCTGGTGT CTAAACTGGA

      240        250        260        270        280        290
   *   *   *   *   *   *   *   *   *   *   *   *
CTCTGGAGTC CCTGACAGGT TCACTGGCAG TGGATCAGGG ACAGATTTCA CACTGAAAAT

      300        310        320        330        340        350
   *   *   *   *   *   *   *   *   *   *   *   *
CAGCAGAGTG GAGGCTGAGG ATTTGGGAGT TTATTATTGC TGCCAAGGTA CACATTTTCC

T
```

FIG. 27C

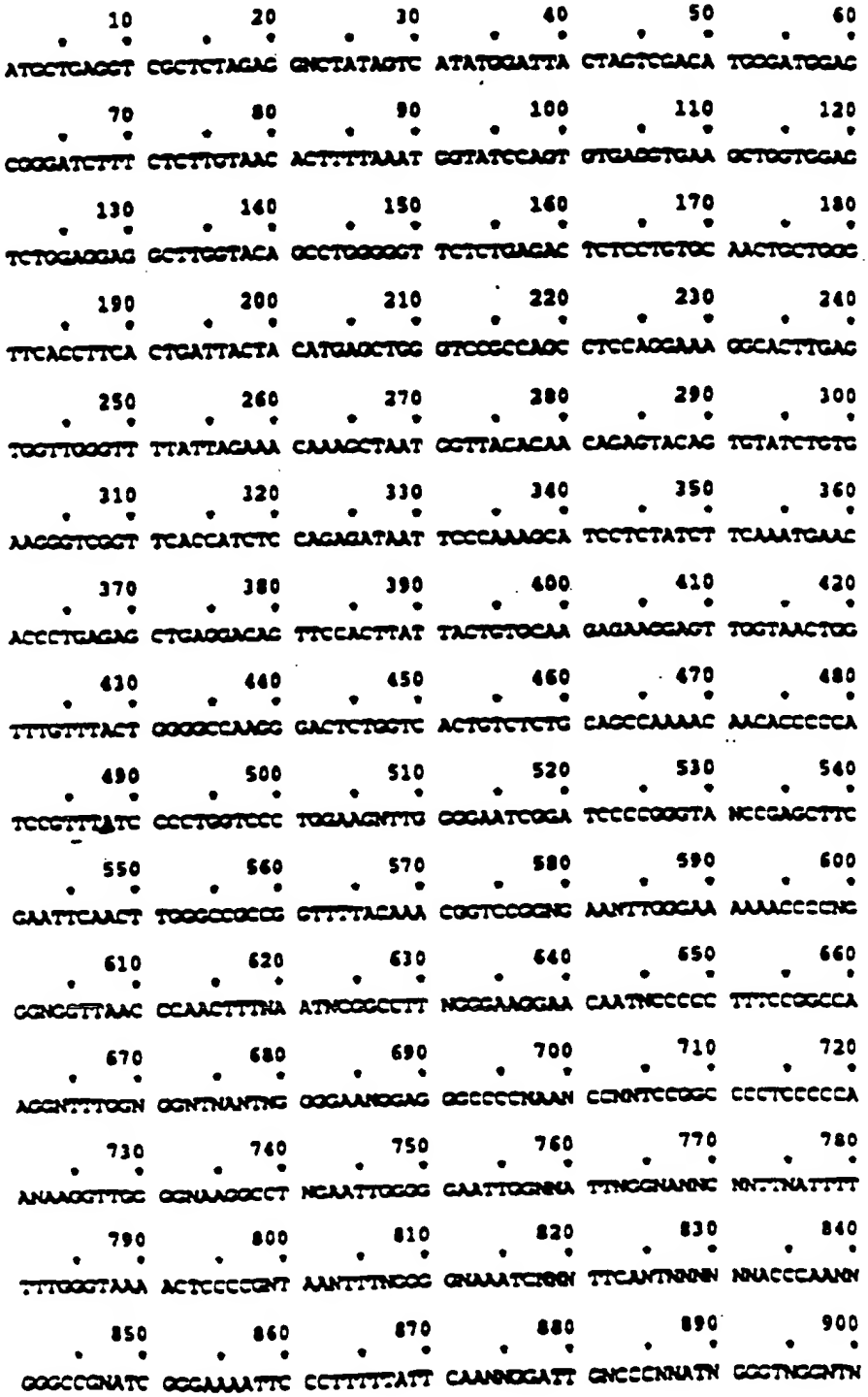


FIG. 27D

```
      10        20        30        40        50        60
      .  .      .  .      .  .      .  .      .  .      .  .
MLRSL*RXIV IWITSRHGME RDLSLVTLLN CIQCEVKLVE SGGGLVQPGG SLRLSCATAG

      70        80        90       100       110       120
      .  .      .  .      .  .      .  .      .  .      .  .
FTFTDYYMSW VRQPPGKALE WLGFIRNKAN GYTTEYSVSV KGRFTISRDN SQSILYLQMN

     130       140       150       160       170       180
      .  .      .  .      .  .      .  .      .  .      .  .
TLRAEDSSTY YCARDGVGNW FVYWGQGTLV TVSAAKTTPP SVYPLVPGXL GNRIPGXPSF

     190       200       210       220       230       240
      .  .      .  .      .  .      .  .      .  .      .  .
EFNLGRRFYK RSGEXGKKPX GG*PNFXXGL XEGTXPPFRP RXWXXXXGXE GPXXXPAPPP

     250       260       270       280       290       300
      .  .      .  .      .  .      .  .      .  .      .  .
XRLGXGLELG ELXIXXXXXF PG*NSPXXXG XNYXXXXXPX GPXRENSLFI QXDXPXXGXX

     310
      .  .
LXPXGXXXPX LXX
```

FIG. 27E

```
      10         20         30         40         50         60
TNANCGAGGC AGTGATTCGA GTCGGTACCG GGGATCCGAT ACTAGTCGAC ATGGGATGGA

      70         80         90        100        110        120
GCTGGATCTT TATCTTCCTC CTGTCAGTAA ATGCAGGTGT CCAAGCCCAG GTTCAATTGC

     130        140        150        160        170        180
AGCAGTCTGG GGCTGAGCTG GTGAGGCCTG GGGCTTCAGT GACGCTGTCC TGCAAGGCTT

     190        200        210        220        230        240
CGGGCTACAC ATTTATTGAG TATGAAATGC ACTGGGTTAA GCAGGCACCT GTGCATGGCC

     250        260        270        280        290        300
TGGAATGGAT TGGAGCTGTT GATCCTGAAA CCGGTGGTAC TGCCTACAAT CAGAAGTTCA

     310        320        330        340        350        360
AGGGCAAGGC CATACTGACT GCAGACAAAT CCTCCAGCAC AGGCTACATG GAGCTCCGCA

     370        380        390        400        410        420
GCCTGACATC TGAAAGTTCT GCCGTCTACT ACTGTGCACG ACAAGGATTA CGTTACTGGG

     430        440        450        460        470        480
GCCAAGGGAC TCTGGTCACT GTCTCTGCAG CCAAAACGAC ANCCCCACCC GTTTATCCCC

     490        500        510        520        530        540
TGGTCCCTGG AAGCTTGGGA TCCATATGAC TAGTAGGATC CTCTAGAGTC GACCTGCAGG

     550        560        570        580        590        600
CATGCAAGCT TTCCCTATAG TGAGTCGNNT TAGAGCTTGG CGTAATCATG GTCATAGCTT

     610        620        630        640        650        660
GTTTCCTGTG NTGAAATTGG TTATCCGNTC AACAATTCCA CACAACATA CGAGCCGGAA

     670        680        690        700        710        720
GCATAANGTG TTAAGNCTGG GGTGCCTAAT GAGTTGACTT ACTCACATTA ATTGCGTGCG

     730        740        750        760        770        780
GNCAATTGCC CGTTNCCAGG CGGCAAACCT NTCGGGCCAG TTGAATTAAT GANTCCGNCC

     790        800        810        820        830        840
ACCCCGGGGN NAGGGGGNTG GGTTTTTGGN NGNTCTTCCC NTTCCTCCGN TCAATTNATT

     850        860        870
CGTTGNNTCG GNCGGNCGGG TTNNGGGGAN GGGNTTNA
```

FIG. 27F

```
        10        20        30        40        50        60
XEAVIRVGTG DPILVDMGWS WIFIFLLSVN AGVQAQVQLQ QSGAELVRPG ASVTLSCKAS
                                                  PA1
        70        80        90       100       110       120
GYTFIEYEMH WVKQAPVHGL EWIGAVDPET GGTAYNQKFK GKAILTADKS SSTGYMELRS
                  PA2                                     PA3
       130       140       150       160       170       180
LTSEDSAVYY CARQGLGYWG QGTLVTVSAA KTTXPPVYPL VPGSLGSI°L VGSSKVDLQA
                            PA4
       190       200       210       220       230       240
CKLSL°°VXL ELGVIMVIAC FLX°NWLSXQ QFHTTIRAGS IXC°IWGA°° VDLLTLIGWG
       250       260       270       280       290
QLPVXRRETX RAS°INXSXH PGXRGXGFWX XFPFLRSIXS LXRXXRXXGX XX
```

FIG. 27G

```
      10         20         30         40         50         60
      *  *       *  *       *  *       *  *       *  *       *  *
ATGAAGTTGC CTGTTAGGCT GTTGGTGCTG ATGTTCTGGA TTCCTGTTTC CAGCAGTGAT

      70         80         90        100        110        120
      *  *       *  *       *  *       *  *       *  *       *  *
GTTGTGATGA CCCAAACTCC ACTCTCCCTG CCTGTCAGTC TTGGAGATCA AGGCTCCATC

     130        140        150        160        170        180
      *  *       *  *       *  *       *  *       *  *       *  *
TCTTGCAGAT CTAGTCAGAG CCTTGTACAC AGAAATGGAA ACACCTATTT ACATTGGTTC

     190        200        210        220        230        240
      *  *       *  *       *  *       *  *       *  *       *  *
CTGCAGAAGC CAGGCCAGTC TCCAAAACTC CTGATCTACA AAGTTTCCAA CCGATTTTCT

     250        260        270        280        290        300
      *  *       *  *       *  *       *  *       *  *       *  *
GGGGTCCCAG ACAGGTTCAG TGGCAGAGGA TCAGGGACAG ATTTCACACT CAAGATCAGC

     310        320        330        340        350        360
      *  *       *  *       *  *       *  *       *  *       *  *
AGAGTGGAGG CTGAGGATCT GGGAGTTTAT TTCTGCTCTC AAAGTAGACA TGTTCCGCTC

     370        380        390        400        410        420
      *  *       *  *       *  *       *  *       *  *       *  *
ACGTTCGGTG CTGGGACCAA GCTGGAGCTG AAACGGGCTG ATGCTGNACC AACTGTATCC

     430        440
      *  *       *  *       *
ATCTTCCCAC CATCCAGTAA GNTTGGG
```

FIG. 27H

```
 60         70         80         90         100        110
  *     *    *     *    *     *    *     *    *     *    *
CAG GTCCAGGTGC AGCAGTCTGG AACTGAACTG GTAAGGCCTG GGACTTCAGC

       120        130        140        150        160        170
    *     *    *     *    *     *    *     *    *     *    *     *
GAAGGTGTCC TGCAAGGCTT CTGGATACGT CTTCTCTACT TACTTGATAG AGTGGATAAA

       180        190        200        210        220        230
    *     *    *     *    *     *    *     *    *     *    *     *
ACAGAGGCCT GGACAGGGTC TTGAGTGGAT TGGGGTGATT AATCCTGGAG GTGGTGGTAT

       240        250        260        270        280        290
    *     *    *     *    *     *    *     *    *     *    *     *
TGACTACAAT GAGAAGTTCA AGGGCAAGGC AACACTGACT GCAGACAAGT CCTCCAGCAC

       300        310        320        330        340        350
    *     *    *     *    *     *    *     *    *     *    *     *
TGCCTACATG CAGCTCAGCA GCCTGACATC TGATGACTCT GCGGTCTATT TCTGTGCAAG

       360        370        380        390        400        410
    *     *    *     *    *     *    *     *    *     *    *     *
ATACACAGAC TATGCTATGG ACTACTGGGG TCAAGGAACC TCAGTCACCG TCTCCTCAGC

CCAA
```

FIG. 27I

```
        10         20         30         40         50         60
    *   *   *   *   *   *   *   *   *   *   *   *
MKLPVRLLVL MFWIPVSSSD VVMTQTPLSL PVSLGDQGSI SCRSSQSLVH RNGNTYLHWF
        70         80         90         100        110        120
    *   *   *   *   *   *   *   *   *   *   *   *
LQKPGQSPKL LIYKVSNRFS GVPDRFSGRG SGTDFTLKIS RVEAEDLGVY FCSQSRHVPL
       130        140
    *   *   *   *   *
TFGAGTKLEL KRADAXPTVS IFPPSSKXG
```

FIG. 27J

```
          10        20        30        40        50        60
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
QVQVQQSGTE LVRPGTSAKV SCKASGYVFS TYLIEWIKQR PGQGLEWIGV INPGGGGIDY

          70        80        90       100       110
   *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
NEKFKGKATL TADKSSSTAY MQLSSLTSDD SAVYFCARYT DYAMDYWGQG TSVTVSSAQ
```

FIG. 27K

```
      10         20         30         40         50         60
      *  *       *  *       *  *       *  *       *  *       *  *
ATGAGGACTT CGATTCAGTT CCTGGGGCTC TTGTTGTTCT GGCTTCATGG TGCTCAGTGT

      70         80         90        100        110        120
      *  *       *  *       *  *       *  *       *  *       *  *
GACATCCAGA TGACACAGTC TCCATCCTCA CTGTCTGCAT CTCTGGGAGG CAAAGTCACC

     130        140        150        160        170        180
      *  *       *  *       *  *       *  *       *  *       *  *
ATCACTTGCA AGGCCAGCCA AGACATTTAC AAGTATATAG CTTGGTACCA ACACAAGCCT

     190        200        210        220        230        240
      *  *       *  *       *  *       *  *       *  *       *  *
GGAAAAGGTC CTAGGCTGCT CATCCATTAC ACATCTACAT TACAGCCAGG CATCCCATCA

     250        260        270        280        290        300
      *  *       *  *       *  *       *  *       *  *       *  *
AGGTTCAGTG GAAGTGGGTC TGGGAGAGAT TATTCCTTCA GCATCAGCAA CCTGGAGCCT

     310        320        330        340        350        360
      *  *       *  *       *  *       *  *       *  *       *  *
GAAGATATTG CAACTTATTA TTGTCTACAG TATGATAATC TGTGGACGTT CGGTGGAGGC

     370        380        390        400        410        420
      *  *       *  *       *  *       *  *       *  *       *  *
ACCAAGCTGG AAATCAAACG GGCTGATGCT GCACCAACTG TATCCATCTT CCCACCATCC

     430
      *  *
AGTAAGCTTG GG
```

FIG. 27L

```
        10         20         30         40         50         60
    *   *   *   *   *   *   *   *   *   *   *   *
MRTSIQFLGL LLFWLHGAQC DIQMTQSPSS LSASLGGKVT ITCKASQDIY KYIAWYQHKP

        70         80         90        100        110        120
    *   *   *   *   *   *   *   *   *   *   *   *
GKGPRLLIHY TSTLQPGIPS RFSGSGSGRD YSFSISNLEP EDIATYYCLQ YDNLWTFGGG

       130        140
    *   *   *   *
TKLEIKRADA APTVSIFPPS SKLG
```

FIG. 27M

```
        10         20         30         40         50         60
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
ATGAACTTTG GGCTCAGCTG GATTTTCCTT GTCCTTGTTT TAAAAGGTGT CCAGTGTGAA

        70         80         90        100        110        120
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
GTGAAACTGG TGGAGTCTGG GGGAGGCTTA GTGAAGCCTG GAGGGTCCCT GAAACTTTCC

       130        140        150        160        170        180
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
TGTGCAGCCT CTGGATTCAG TTTCAGTAGC TATGCCATGT CTTGGGTTCG CCAGACTCCA

       190        200        210        220        230        240
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
GAGAAGAGGC TGGAGTGGGT CGCATCCATT AGTAGTGGTG GTAACACCTA CTATCCAGAC

       250        260        270        280        290        300
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
AGTGTGAAGG GCCGATTCAC CATCTCCAGA GATAATGCCA GGAACATCCT GTACCTGCAA

       310        320        330        340        350        360
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
ATGAGCAGTC TGAGGTCTGA GGACACGGCC ATGTATTACT GTGCAAGATT AGATACTACG

       370        380        390        400        410        420
    *   *  *    *   *  *    *   *  *    *   *  *    *   *  *    *   *
GTAGAAGGGG ACTGGTACTT CGATGTCTGG GGCGCAGGGA CCAGCCTCAC CGTCTCCTCA

       430        440        450        460
    *   *  *    *   *  *    *   *  *    *   *
GCCCAAACAA CACCCCCACC CGTCTATCCC TTGGTCCCTG G
```

FIG. 27N

```
        10        20        30        40        50        60
    *   *   *   *   *   *   *   *   *   *   *   *
MNFGLSWIFL VLVLKGVQCE VKLVESGGGL VKPGGSLKLS CAASGFSFSS YAMSWVRQTP

        70        80        90       100       110       120
    *   *   *   *   *   *   *   *   *   *   *   *
EKRLEWVASI SSGGNTYYPD SVKGRFTISR DNARNILYLQ MSSLRSEDTA MYYCARLDTT

       130       140       150
    *   *   *   *   *   *
VEGDWYFDVW GAGTSLTVSS AQTTPPPVYP LVP
```

FIG. 270

INTERNATIONAL SEARCH REPORT

Int[redacted]al application No.
PCT/US96/07393

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 48/00 39/42; C12N 15/86
US CL :435/172.3, 320.1; 424/147.1, 148.1,135.1, 93.2, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/172.3, 320.1; 424/147.1, 148.1,135.1, 93.2, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

DIALOG DATABASES: BIOSIS, MEDLINE, WORLD PATENT INDEX, AIDSLINE. APS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	DUAN, L. et al. Potent inhibition of human immunodeficiency virus type 1 replication by an intracellular anti-Rev single-chain antibody. Proc. Natl. Acad. Sci. USA. May 1994, Vol. 91, pages 5075-5079, see entire article.	30,34,35 --- 1-29,31-33,35
X --- Y	CHEN, S.-Y. et al. Combined intra- and extracellular immunization against human immunodeficiency virus type 1 infection with a human anti-gp120 antibody. Proc. Natl. Acad. Sci. USA. June 1994, Vol. 91, pages 5932-5936, see entire article.	30,34 --- 1-29,31-33, 35

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

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"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 15 JULY 1996	Date of mailing of the international search report 14 AUG 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JOHNNY F. RAILEY II, PH.D. Telephone No. (703) 308-0196

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C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	FARAJI-SHADAN, F. et al. A Putative Approach for Gene Therapy Against Human Immunodeficiency Virus (HIV). Medical Hypotheses. 1990, Vol. 32, pages 81-84, see entire article.	1-35
Y	BALTIMORE, D. Gene Therapy: Intracellular Immunization. Nature. 29 September 1988, Vol. 335, pages 395-396, see entire article.	1-35
Y	BIOCCA, S. et al. Expression and targeting of intracellular antibodies in mammalian cells. The EMBO Journal. 1990, Vol. 9, No. 1, pages 101-108, see entire article.	1-35
Y	MUZYCZKA, N. Use of Adeno-Associated Virus as a General Transduction Vector for Mammalian Cells. Curr. Top. Microbiol. Immunol. 1992, Vol. 158, pages 97-129, see entire article.	32
Y	PAUL, N. L. et al. Expression of HIV-1 Envelope Glycoproteins by Semliki Forest Virus Vectors. AIDS Research and Human Retroviruses. October 1993, Vol. 9, No. 10, pages 963-970, see entire article.	33